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Identification of a sporulation gene in Bacillus thuringiensis using transposon-mediated insertional mutagenesis

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IDENTIFICATION OF A SPORULATION GENE IN *BACILLUS THURINGIENSIS* USING TRANSPOSON-MEDIATED INSERTIONAL MUTAGENESIS

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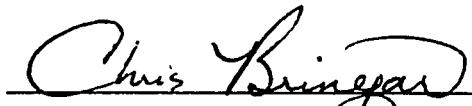

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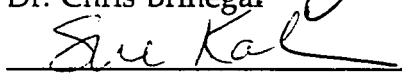
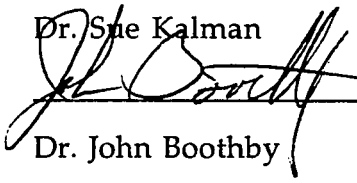


Dr. Sue Kalman



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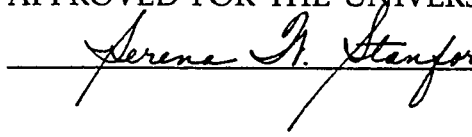

ABSTRACT

IDENTIFICATION OF A SPORULATION GENE IN *BACILLUS THURINGIENSIS* USING TRANSPOSON-MEDIATED INSERTIONAL MUTAGENESIS

by Mitra Shahabi Reynoso

Bacillus thuringiensis has been used extensively as a biological insecticide to control a wide range of insect pests. Under conditions of nutrient limitation, this organism undergoes sporulation which is a unicellular differentiation process. Numerous genes responsible for different stages of spore formation have been characterized in *Bacillus* species. However, only a few sporulation genes have been identified in *Bacillus thuringiensis*.

Using transposon Tn917 insertional mutagenesis, a new sporulation gene was identified in *Bacillus thuringiensis*. Approximately 70% identity at both the DNA and the protein level was observed between the isolated sporulation gene and the *spoVJ* gene from *Bacillus subtilis*. The *spoVJ* mutant of *Bacillus thuringiensis* produced immature spores that were sensitive to heat and organic solvents but resistant to lysozyme. These phenomena were also observed in the *Bacillus subtilis spoVJ* mutant. Based on these results, it is concluded that the isolated gene is indeed the *spoVJ* gene from *Bacillus thuringiensis*.

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Special thanks are due to Dr. Sue Kalman for her endless patience, guidance, and support during my thesis project, to thesis committee members Dr. Chris Brinegar and Dr. John Boothby for their contributions, to Nicole Sinibaldi for helping me during moments of crisis and being so patient, to Gary Powell for providing computer support when I needed it, to Dave Matthews for preparing the oligonucleotides needed for sequencing, and to Sandoz Agro Inc. for allowing me to conduct my research in their facilities.

Finally, I would like to acknowledge the support I received from my husband, Sam, who helped make my thesis-related stress a little more bearable, and from my parents who taught me the value of a good education.

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INTRODUCTION

Sporulation, a survival mechanism available to some bacteria in response to extreme environmental conditions, involves a radical change of the biosynthetic activities in the bacterium. The genes that are needed for sporulation can be recognized by creating mutations which permit normal vegetative growth, but block sporulation. Sandman *et al.* (1987) performed a genetic analysis of *Bacillus subtilis* sporulation mutants produced by transposon-mediated insertional mutagenesis. The transposon used was the *Streptococcus faecalis* transposon Tn917. In Sandman's study, the insertion mutations blocked sporulation at various stages and therefore identified genes active at different times during sporulation.

Bacillus thuringiensis, which has some of the same phenotypic properties as *B. subtilis*, produces crystalline protein bodies in addition to endospores during sporulation. The crystals from different strains of *B. thuringiensis* are toxic to several orders of insects, such as larvae of Lepidoptera and Coleoptera. *B. thuringiensis* has been of great interest to many researchers due to its importance in the biological control of these agricultural pests. Although many studies have been done on the crystal proteins from *B. thuringiensis*, little is known about the sporulation genes. These genes are not only associated with crystal production but are also responsible for sporulation, a process used by this organism to survive under certain conditions of nutrient limitation.

In the current study, the transposon-mediated insertional mutagenesis technique was used to identify genes involved in spore formation in *B.*

thuringiensis subspecies *kurstaki* HD1Mit9. A sporulation mutant was created using transposon Tn917. The gene interrupted with Tn917 was isolated, cloned into an *Escherichia coli* strain, sequenced, and compared to the known sporulation genes in *B. subtilis* using computer analysis.

LITERATURE REVIEW

B. thuringiensis is a gram-positive, aerobic, spore-forming bacterium that can produce crystalline inclusions during sporulation. The inclusions are composed of one or more insecticidal proteins which are toxic to certain insect larvae (Cooper, 1994). Insecticidal proteins toxic to lepidopteran, coleopteran, and dipteran insect larvae have been characterized (Whiteley and Schnepf, 1986). Over the past few decades, *B. thuringiensis*-based products have been widely used for the control of agricultural and forest pests. One important advantage of using biological insecticides is that they have the ability to destroy pests resistant to chemicals (Watkinson, 1994). Many countries, including Australia and the USA, use *B. thuringiensis*-based products for the control of diamondback moth resistant to pyrethroids.

B. thuringiensis was first isolated by the Japanese bacteriologist S. Ishiwata in 1901 from a diseased silkworm larvae (Beegle and Yamamoto, 1992). Because the isolated *Bacillus* strain was extremely pathogenic to larval silkworm, he named it Sottokin which means "sudden death bacillus". In 1915, Berliner isolated a similar *Bacillus* from a diseased Mediterranean flour moth from Thuringia, Germany, and named it *B. thuringiensis* (Beegle and Yamamoto, 1992). Studies done by Aoki and Chigasaki suggested that the toxicity of this organism was due to the presence of a protein found in sporulated cultures.

In 1951, Edward Steinhaus at the University of California at Berkeley, published an article encouraging the commercial use of *B. thuringiensis* as a biological control agent for lepidopteran insect pests (Beegle and Yamamoto,

1992). Since his work in the early 1950's, various *B. thuringiensis* products have been used in most countries of the world for control of caterpillars, mosquitoes, blackfly, and beetle larvae (Milner, 1994).

Bacillus species undergo dramatic morphological, physiological, and biochemical changes during sporulation (Errington, 1993). These changes have been studied in great detail in *B. subtilis*. In general, sporulation is used as a terminal differentiation pathway for the organism under certain nutrient-limiting conditions. Once sporulation is initiated, the cells undergo seven morphological stages (Losick *et al.*, 1986). Vegetative cells are said to be in stage 0. As sporulation begins, the chromosome condenses into an axial filament. This event has been defined as stage I, but was disputed until recently because the event is not specific to sporulation and no mutations had been found in *B. subtilis* which block sporulation at this stage (Piggot and Coote, 1976). However, Tao and Vary (1991) have recently characterized a *Bacillus megaterium* sporulation mutant defective in stage I of development. At stage II, cell division occurs producing two cells that are different in size. The smaller cell is the daughter cell also known as forespore or prespore, and the larger cell is designated the mother cell. During stage III, the mother cell engulfs the forespore resulting in the formation of a double membrane around the forespore inside the mother cell. A modified form of a cell wall known as cortex is synthesized between the inner and outer membranes of the prespore during stage IV. Spore coat deposition on the outer membrane of the prespore occurs during stage V. Stage VI is defined as the complete maturation of the spore. At this stage the spore develops its characteristic properties of resistance to radiation, heat, lysozyme, and organic solvents.

Finally, the mother cell lyses and the mature spore is released in stage VII. The free spore is refractile and can be easily observed using light microscopy.

Because of the considerable interest in the use of *B. thuringiensis* as a biological pesticide, numerous studies had been done to identify insecticidal proteins in this bacterium. However, little is known about the genes involved in the sporulation pathway of this organism. Most insecticidal crystal proteins are expressed only during sporulation (Beegle and Yamamoto, 1992). Because of this, it is also important to identify and characterize the genes involved in the sporulation pathway so that crystal production and sporulation can be manipulated.

One useful approach to identifying sporulation-associated genes is transposon-mediated insertional mutagenesis (Youngman, 1987). A transposon is a mobile segment of DNA that is flanked by terminal repeat sequences (Grindley and Reed, 1985). It is able to insert itself at a new site in the bacterial chromosome or plasmid, a process called transposition. Transposons also encode the proteins necessary for their transposition and some also carry an antibiotic resistance gene. If the insertion occurs within a gene, the protein encoded by that gene would most likely become nonfunctional.

In the current study, a sporulation gene was isolated from a *B. thuringiensis* strain using insertional mutagenesis with the *S. faecalis* transposon Tn917 (Tomich *et al.*, 1980). Malvar and Baum (1994) used the same approach with a different transposon, Tn5401, to isolate the *B. thuringiensis spo0F* gene. In 1990, Crawford and Streips described a procedure to isolate *B. thuringiensis* sporulation mutants through Tn917 insertional

mutagenesis. However, they did not report the characterization of any particular sporulation genes.

Tn917 had been used extensively in mutagenesis studies for *B. megaterium* and *B. subtilis*. Bohall and Vary (1986) obtained sixty-nine auxotrophs and forty-three sporulation mutants from *B. megaterium* using Tn917 insertional mutagenesis. They identified eight auxotrophic and two carbon source loci. However, the exact location of the sporulation mutations was not determined. In 1991, Tao and Vary characterized four *B. megaterium* insertion mutants defective in stages I and II of sporulation. Sandman *et al.* (1987) described the genetic and phenotypic characterization of twenty-four Tn917 insertional mutations in *B. subtilis* that blocked sporulation at different stages including 0, II, III, IV, and V. Vandeyar and Zahler (1986) isolated and mapped twenty-seven auxotrophic and nineteen cryptic insertions of Tn917 in the *B. subtilis* chromosome. In 1983, Youngman *et al.* recovered different kinds of auxotrophic and sporulation-defective *B. subtilis* mutants from a Tn917 insertion library. Approximately 1-2% were auxotrophs and 0.1-0.5% were sporulation mutants. Youngman found three sporulation mutants that blocked development at stage II or III of the sporulation pathway, based on their phenotypic characteristics. Tn917 was also used to identify genes involved in pathogenicity of *Listeria monocytogenes* (Camilli *et al.*, 1990).

According to Youngman *et al.* (1983), the transposition frequency of Tn917 in *B. subtilis* was equivalent to that in *Streptococcus* species. It was also shown that the distribution of Tn917 insertions in the *B. subtilis* chromosome was sufficiently random. As a result, transposon Tn917 was

chosen for isolation of a *B. thuringiensis* sporulation gene through mutagenesis.

In the current study, *B. thuringiensis* strain HD1Mit9 was used for isolation of a sporulation gene. This strain does not produce insecticidal proteins. HD1Mit9 was chosen because it contains only one plasmid and it is a derivative of *B. thuringiensis* subspecies *kurstaki* HD1, the most well-studied *B. thuringiensis* strain. HD1 was isolated from diseased *Pectinophora gossypiella* larvae by Dulmage (1970). Later, HD1 was found to belong to a new subspecies of *B. thuringiensis*, which was named *kurstaki* (de Barjac and Lemille, 1970).

MATERIALS AND METHODS

Bacterial Strains

The *B. thuringiensis* HD1Mit9 strain was obtained from Dr. Arthur I. Aronson (Purdue University). Cry$^-$B, a crystal-minus cured strain of *B. thuringiensis*, was received from Repligen Sandoz Research Corporation (Lexington, MA). Plasmid pLTV1, which contains transposon Tn917, was isolated from the *B. subtilis* strain PY1177 obtained from Dr. Philip Youngman (University of Pennsylvania School of Medicine). The *E. coli* strains DH5α [F$^-$ ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-argF)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-,m_K+) *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1] from Gibco BRL (Grand Island, NY), GM2163 [F$^-$ *ara*$^-$14 *leu*B6 *ton*A31 *lac*Y1 *tsx*$^-$78 *sup*E44 *gal*K2 *gal*T22 *his*G4 *rps*L136 *xyl*$^-$5 *mtl*$^-$1 *thi*$^-$1 *dam*$^-$13 Tn9 (Cr^r) *dcm*$^-$6 *hsd*R2 *mcr*B$^-$ *mcr*A$^-$] from New England Biolabs, Inc. (Beverly, MA), and HB101 [F$^-$ *mcr*B *mrr* *hsd*S20($r_B^-m_B^-$) *rec*A13 *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*5$^-$ λ$^-$ *leu mtl*1] from New England Biolabs, Inc. were used as hosts for plasmid DNA in this study.

Media, Materials, and Bacterial Growth

Phage CP-51ts45 used for transduction was obtained from Dr. Curtis B. Thorne (University of Massachusetts at Amherst). The restriction enzymes, T4 DNA Ligase, and reaction buffers were purchased from New England Biolabs, Inc., Pharmacia Biotechnology (Piscataway, NJ), or Gibco BRL. The antibiotics such as ampicillin (Amp), chloramphenicol (Cm), erythromycin (Ery), lincomycin (Lm), tetracycline (Tet), and chemicals were obtained from

Sigma Chemical Company (St. Louis, MO). The oligonucleotides used for the polymerase chain reaction (PCR) and sequencing were synthesized on a PCR-Mate DNA synthesizer model 391 (Applied Biosystems, Foster City, CA) by Dave Matthews at Sandoz Agro, Inc. (Palo Alto, CA).

The *E. coli* and *B. thuringiensis* strains were grown in L-broth (LB) medium (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 0.5% NaCl, pH 7.0) at 37°C and 30°C respectively, unless otherwise stated. Solid media were prepared using 15 g Bacto agar per liter. High efficiency *E. coli* competent cells used for electroporation were made using SOB medium (2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 0.06% NaCl, 0.05% KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$). After electroporation, *E. coli* cells were grown in SOC medium (2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 0.06% NaCl, 0.05% KCl, 20 mM glucose). SA plates (1x Spizizens salts, 1% casamino acids, 0.5% glucose, 0.005 mM $MnSO_4.H_2O$, 1.5% Bacto agar) were used for *B. thuringiensis* plasmid isolation. The 1x Spizizens salts were made of 0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% Sodium-Citrate·$2H_2O$, and 0.02% $MgSO_4 \cdot 7H_2O$ (Anagnostopoulos and Spizizen, 1961). BHIS medium (3.7% Brain Heart Infusion, 0.5 M sucrose) was used for preparation of *B. thuringiensis* competent cells. Penassay broth (1.75% Difco Antibiotic Medium 3) was used for preparation of Tn917 insertion libraries. *B. thuringiensis* colonies with transposon insertions were grown on CYS plates (1% Casitone, 0.5% glucose, 0.2% Bacto Yeast Extract, 0.1% KH_2PO_4, 0.5 mM $MgCl_2$, 0.05 mM $MnCl_2$, 0.05 mM $ZnSO_4$, 0.05 mM $FeCl_2$, 0.2 mM $CaCl_2$, 1.5% Bacto agar) to test their ability to sporulate. Phage Assay (PA) medium (0.8% nutrient broth, 0.5%

NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot H_2O$, 0.015% $CaCl_2 \cdot 2H_2O$, pH 6.0) was used for preparation of phage lysates in transduction experiments.

To identify auxotrophs and citric acid cycle-defective colonies, the *B. thuringiensis* sporulation mutants were grown on glucose minimal and lactate minimal plates for 1-2 days, and the growth was compared to that of HD1Mit9. The glucose minimal agar was made of 1x salt solution [1 liter; 5.6 g K_2HPO_4, 2.4 g KH_2PO_4, 1.2 g $(NH_4)_2SO_4$, 0.4 g sodium citrate, pH 7.0], 0.005 mM $FeCl_2$, 0.25 mM $MgCl_2$, 0.96% glucose, 0.0002 mM $MnCl_2$, 0.00012% Thiamine-B1, and 1.5% Bacto agar. The lactate minimal medium contained 0.2% lactate and 25 mM glutamate instead of glucose and sodium citrate.

The expression of the *lacZ* gene in transposon Tn917 was examined by growing the strains carrying the transposon on LB agar plates containing Ery^5 (erythromycin at 5 µg/ml) and 60 µg/ml of 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal) for five days, and observing the colonies for the presence or absence of blue color. To determine the growth rates of the *B. thuringiensis* strains, cells were grown overnight on CYS plates and single colonies were used to inoculate 100 ml of CYS medium in a 500 ml flask. The cultures were incubated at 30°C and 300 revolutions per minute (rpm). The growth was monitored by optical density at 600 nm (OD_{600}) every half hour until the transition from exponential growth into stationary phase occurred.

Isolation of DNA from *Bacillus* and *E. coli* Strains

***B. subtilis* plasmid preparation.** The strain was grown overnight (18-20 hours) on TBAB plates (3.3% Difco Tryptose Blood Agar Base) containing 0.5% glucose and Tet^{10}. A single colony was used to inoculate 10 ml LB

containing Tet^{10} and 0.5% glucose. The cells were grown for 5 hours at 300 rpm, centrifuged at 18,500 x g and 4°C for 10 minutes, then washed once in 10 ml of SET buffer [20% sucrose, 50 mM disodium ethylenediaminetetra-acetate·$2H_2O$ (EDTA), 50 mM Tris-HCl pH 8.0]. The pellet was then resuspended in 500 µl SET solution containing 2 mg/ml of lysozyme and 0.4 mg/ml RNase A (Boehringer Mannheim Biochemicals, Indianapolis, IN). The cell suspension was incubated at 37°C for 10 minutes and 1 ml of the lytic mix [1% sodium dodecycl sulfate (SDS), 200 mM NaOH] was added, followed by 725 µl of prechilled neutralization buffer (1.5 M potassium acetate pH 4.8). The mixture was then incubated on ice for 20 minutes. The sample was microcentrifuged at 18,500 x g and 4°C for 10 minutes and the supernatant was transferred to a fresh tube. The DNA was then isolated using a Mini Qiagen Plasmid Kit (Qiagen Inc., Chatsworth, CA) as follows. A Qiagen-Tip 20 column was equilibrated with 1 ml QBT equilibration buffer [750 mM NaCl, 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.0, 15% ethanol, 0.15% Triton X-100]. The supernatant was applied to the column and allowed to enter the resin by gravity flow. The Qiagen column was washed with 2 ml QC wash buffer (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% ethanol), and the DNA was eluted using 0.8 ml QF elution buffer (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% ethanol). The DNA was precipitated using 500 µl isopropanol and microcentrifuged at room temperature for 25 minutes at 16,000 x g. The DNA pellet was washed in prechilled 70% ethanol and then resuspended in 20 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) or sterile deionized distilled water (ddH_2O). For long term storage, the DNA was kept at -20°C.

***B. thuringiensis* plasmid DNA isolation.** HD1Mit9 and Cry$^-$B were streaked onto LB agar plates, and HD1Mit9/pLTV1 and Cry$^-$B/pLTV1 (the *B. thuringiensis* strains containing plasmid pLTV1) onto LB Tet^{10} plates. The cultures were grown overnight, and a single colony from each strain was restreaked onto an SA plate and incubated for 3-4 hours at 37°C. The grown cells were removed from the plate and resuspended in 100 µl TESL (100 mM Tris-HCl pH 8.0, 10 mM EDTA, 20% sucrose, 2 mg/ml lysozyme), and incubated at 37°C for 30-60 minutes. Two hundred microliters of lysis solution (200 mM NaOH, 1% SDS) were added to each tube followed by a 5 minute incubation at room temperature. After addition of 150 µl ice-cold 3 M potassium acetate pH 4.8, the suspension was microcentrifuged for 20 minutes at 18,500 x g and 4°C. The supernatants were transferred to fresh tubes and mixed with 1 ml of 100% ethanol. This suspension was left at -20°C for 1 hour and centrifuged at 18,500 x g and 4°C for 30 minutes. The plasmid DNA was washed in 70% ethanol, dried under vacuum, and resuspended in 20 µl of TE.

Total DNA isolation from *B. thuringiensis*. The strain was grown in 2 ml LB overnight at 200 rpm. The overnight culture was used to inoculate 100 ml LB (1% inoculation). The cells were grown at 300 rpm to an OD_{600} of 0.7-1.0, collected by centrifugation (3,840 x g, 5 minutes, 4°C), and resuspended in 5 ml TES (25 mM Tris-HCl pH 8.0, 25 mM EDTA, 25% sucrose). The cell suspension was mixed with 0.55 ml of 10 mg/ml lysozyme in TES solution and incubated at 37°C for 60 minutes. SDS was added to 2% weight-to-

volume, followed by a 15 minute incubation at 50°C. The suspension was then mixed with 1.52 ml of 5 M NaCl and incubated at 50°C for 5 minutes. The sample was incubated at 0°C for 1 hour, centrifuged for 10 minutes at 18,500 x g and 4°C, and the supernatant was transferred to a new tube. To purify the DNA from the protein, the supernatant was treated with phenol and chloroform. First, it was mixed with 5 ml TE-equilibrated phenol followed by 15 minutes incubation at 50°C, then with 5 ml phenol/chloroform (1:1), and finally with 5 ml chloroform. The aqueous phase was separated from the organic phase by centrifugation (4,000 x g, 5 minutes) at each step. The DNA was precipitated by the addition of 4.6 ml isopropanol and centrifugation (18,500 x g, 30 minutes, 4°C). The air-dried DNA pellet was dissolved in 2 ml of TE and stored at -20°C.

***E. coli* plasmid DNA preparation.** The following protocol was used to isolate plasmid DNA for restriction enzyme digestion. The buffers (P1, P2, and P3) were obtained from Qiagen, Inc. The *E. coli* strain was grown in 4 ml LB containing Amp^{75} at 300 rpm for 18-20 hours. The culture was centrifuged (4,000 x g, 4°C, 10 minutes) and resuspended in 300 µl P1 (100 µg/ml RNase A, 50 mM Tris-HCl pH 8.0, 10 mM EDTA). The cells were lysed using 300 µl P2 (200 mM NaOH, 1% SDS) followed by a 5 minute incubation at room temperature. To precipitate the protein and cell debris, the viscous cell suspension was gently mixed with 300 µl P3 (3 M potassium acetate, pH 5.5) and placed on ice for 10 minutes. The solution was centrifuged for 15 minutes at 8,170 x g, and the supernatant was immediately transferred to a fresh tube. The DNA was precipitated by adding 675 µl isopropanol and

microcentrifuging at 18,500 x g and 4°C for 30 minutes. The DNA pellet was then washed with 70% ethanol, dried under vacuum, and resuspended in 20 µl TE.

The plasmid DNA used for sequencing was isolated from *E. coli* DH5α strain using the Maxi Qiagen Plasmid Kit (Qiagen Inc.). The DNA used for *B. thuringiensis* electroporation experiments was purified from *E. coli* GM2163 using the Midi Qiagen Plasmid Kit.

DNA Methods

Polymerase chain reaction. *B. thuringiensis* cells were used as a PCR template in this study. The cells were grown overnight on a solid medium. A single colony was resuspended in 15 µl sterile water, boiled for 10 minutes, and then centrifuged for 5 minutes. Each PCR reaction contained 2 µl of the supernatant from the boiled cells, 1x PCR buffer [100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% (wt/vol) gelatin], 200 µM deoxyribonucleoside triphosphates (dNTP's; 1.25 mM of dATP, dCTP, dGTP, and dTTP), 1 µM LacNHS1 primer, 1 µM LacNHS2 primer, and 2.5 units *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The oligonucleotide primers were designed by Nicole Sinibaldi at Sandoz Agro, Inc. The reactions were run in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 25 cycles. Each cycle consisted of 1 minute at 94°C (denaturation), 2 minutes at 55°C (hybridization), and 3 minutes at 72°C (extension). The PCR products were analyzed by agarose gel electrophoresis.

Agarose gel electrophoresis. The PCR products and restriction enzyme digests of plasmids were analyzed by horizontal agarose gel electrophoresis. DNA was mixed with 0.1 volume 10x loading dye (0.4% bromophenol blue, 0.4% xylene cyanol, 50% glycerol), loaded onto a 0.8% agarose gel in 1x TBE (1.08% Tris-base, 0.55% boric acid, 2 mM EDTA, pH 8.0) containing 0.5 μg/ml of ethidium bromide, and run on a Submarine Agarose Gel Unit (Hoefer Scientific Instruments, San Francisco, CA). The gel was then photographed on a short wavelength UV box (UVP, Inc., San Gabriel, CA).

To visualize the plasmids in *B. thuringiensis* strains, the plasmid DNA (10 μl) was added to 2 μl of 6x gel loading buffer (0.24% bromophenol blue, 0.24% xylene cyanol, 30% glycerol, 10 μg/ml RNAse), loaded on a vertical gel containing 0.8% agarose in 1x TBE buffer, and run at 30 mA for 3 hours on an Adjustable Slab Gel System (C.B.S. Scientific Company, Inc., Del Mar, CA). The gel was transferred to a staining solution (1x TBE with 0.5 μg/ml ethidium bromide) and incubated with slow shaking for 20 minutes. The gel was destained in water briefly and then photographed on a short wavelength UV box.

DNA sequencing. The Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH) was used to sequence the cloned *B. thuringiensis* chromosomal fragments using the procedure described by the manufacturer. The DNA sequences were then compared to all the known sequences in GenBank using the FastA program of GCG version 7.0 (Genetics Computer Group, Inc., Madison, WI). The GCG program was also used for

translating the DNA sequence and calculating the molecular weight of the protein product.

Preparation of Competent Cells

***B. thuringiensis* competent cells.** The culture was grown overnight on an LB plate. A loopfull of individual colonies from the overnight culture was used to inoculate 100 ml of BHIS medium in a 1 L flask. The culture was grown at 37°C with shaking until it reached an OD_{600} of 0.2-0.3. The cells were transferred to a prechilled 250 ml bottle and kept on ice for the remainder of the experiment. The culture was centrifuged for 7 minutes at 6,480 x g and 4°C. The pellet was washed once in 100 ml and twice in 10 ml of ice cold HEPES [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)]/sucrose wash solution (0.5 M sucrose and 5 mM HEPES at pH 7.0). The cells were then resuspended in 10 ml of the HEPES/sucrose solution and 2.5 ml of 50% glycerol. The competent cells were placed in prechilled 1.7 ml microfuge tubes (400 µl of cells per tube), frozen on dry ice, and kept at -80°C for long term storage.

High efficiency *E. coli* GM2163 electro-competent cells. *E. coli* strain GM2163 was grown overnight on an LB plate. A single colony was used to inoculate 30 ml SOB medium. The cells were grown overnight at 300 rpm. Two hundred milliliters of SOB in a 2 L flask were inoculated with 8 ml of the overnight culture and incubated at 37°C and 300 rpm to an OD_{550} of 0.3. The culture was placed on ice for 15 minutes, centrifuged at 3,840 x g and 4°C for 5 minutes, and the pellet was resuspended in 64 ml of transformation

buffer 1 (1.2% RbCl, 0.99% $MnCl_2 \cdot 4H_2O$, 30 mM potassium acetate pH 5.8, 0.25% $CaCl_2 \cdot 2H_2O$, 15% glycerol). After a 15 minute incubation on ice, the cells were centrifuged at 3,840 x g and resuspended in 16 ml of transformation buffer 2 (10 mM MOPS pH 7.0, 0.12% RbCl, 1.1% $CaCl_2 \cdot 2H_2O$, 15% glycerol). The competent cells were transferred to microcentrifuge tubes, frozen on dry ice, and placed in the -80°C freezer.

***E. coli* DH5α and HB101 competent cells.** The strains were grown on LB agar for 16-18 hours. A few colonies were used to inoculate 100 ml LB in 1L Erlenmeyer flasks. The liquid cultures were grown at 37°C and 300 rpm. Once the cultures reached an OD_{600} of 0.2, they were placed on ice for 15 minutes and then centrifuged for 10 minutes at 5,520 x g and 4°C. The cells were resuspended in 50 ml (1/2 volume) 0.05 M $CaCl_2$, incubated on ice for 20 minutes, and centrifuged as described above. The competent cells were then resuspended in 5 ml (1/20 volume) 0.05 M $CaCl_2$ containing 20% glycerol, placed into microfuge tubes (100 µl per tube), and frozen on dry ice. The cells were stored at -80°C.

B. thuringiensis and *E. coli* Transformation

Electroporation of *B. thuringiensis*. The *B. thuringiensis* competent cells (200 µl) were mixed with 10 µl of DNA (1-5 µg) in a prechilled 0.2 cm gap electrode Gene Pulser Cuvette and exposed to the electrical current in the Gene Pulser electroporation apparatus (Bio-Rad Laboratories, Richmond, CA). This device consists of a pulse controller and a capacitance extender. The parameters for the electroporation of HD1Mit9 were 1.2 kV, 3 µF, $\Omega = \infty$, and

for Cry⁻B were 1.05 kV, 25 μF, Ω = ∞. The cells were immediately transferred to 5 ml BHIS in a 125 ml flask and grown at 30°C and 250 rpm. After three hours of growth, the cells were transferred to LB agar plates containing Tet^{10}. The plates were incubated overnight at 30°C and the transformants were restreaked onto fresh LB Tet^{10} plates.

Electroporation of *E. coli* strain GM2163. The frozen competent cells were thawed at room temperature. Approximately 50 μl of competent cells and 4 μl DNA (1-2 μg) were mixed in a 1.5 ml Eppendorf tube and incubated on ice for 1 minute. The mixture of cells and DNA was transferred to a prechilled 0.2 cm gap electrode cuvette and pulsed in the high voltage Gene Pulser electroporation apparatus. The settings used were 25 μF, 2.5 kV, and 200 Ω. Immediately following the electroporation, the cells were transferred to 1 ml SOC medium in a 17 X 100 mm polypropylene tube (VWR Scientific, San Francisco, CA) and incubated at 37°C and 225 rpm for 1 hour. The cells were then transferred to LB agar containing Amp^{75} and grown overnight.

Transformation of *E. coli* strains DH5α and HB101. The frozen competent cells (100 μl/tube) were thawed at room temperature, mixed with 0.5-1 μg of DNA, and incubated on ice for 20 minutes. The mixture was heated at 42°C for 90 seconds and then placed on ice for 10 minutes. The cells were transferred to 900 μl of LB and incubated at 37°C and 250 rpm for 1 hour. The sample was transferred to LB agar containing Amp^{75} and grown overnight. The transformants were restreaked onto fresh LB Amp^{75} plates.

Determination of pLTV1 Stability in *B. thuringiensis*

The HD1Mit9/pLTV1 strain was grown overnight on an LB plate containing Tet^{10}. Several colonies from the overnight plate were used to inoculate 15 ml LB containing Tet^{10}. The culture was grown at 30°C to an OD_{600} of 0.4. The cells were centrifuged at 7,000 x g and washed in 10 ml LB to remove the antibiotic. One hundred fifty microliters of the washed cells were used to inoculate 15 ml LB in four 125 ml flasks. The cells were grown at four different temperatures, 30°C, 37°C, 40°C, and 42°C to an OD_{600} of 0.6-0.8. Various dilutions of each culture were plated onto LB plates containing $Ery^{0.05}$ and incubated overnight at the same temperatures. The colonies grown on these plates were patched onto LB and LB Tet^{10} plates. The percentage of bacteria in the population that contained the plasmid was determined by dividing the number of colonies which grew on LB Tet^{10} by the total number of colonies.

Preparation of Tn917 Insertion Libraries

The *B. thuringiensis* HD1Mit9 strain containing plasmid pLTV1 was grown on an LB plate containing Tet^{10}. Two to three colonies were used to inoculate 10 ml Penassay broth containing $Ery^{0.15}$. After 90 minutes of growth at 30°C and 300 rpm, the concentrations of antibiotics in the culture were increased to Ery^{1} and Lm^{25}. When the culture reached an OD_{595} of 0.5, a 100 µl portion was added to 10 ml fresh Penassay broth containing Ery^{1} and Lm^{25}. After 16 hours of growth at 39°C and 300 rpm, the culture was diluted 1:15 into 10 ml Penassay broth containing Ery^{1} and Lm^{25} and grown with moderate shaking at 39°C until it reached an OD_{595} of 2.0. The cells were

pelleted by centrifugation (4,000 x g, 4°C), resuspended in 500 µl of Penassay broth containing Ery^1 and 30% glycerol, and frozen on dry ice. The libraries were stored at -80°C.

To identify colonies that contained a Tn917 insertion, the 10^{-3} and 10^{-4} dilutions of the libraries were plated onto Penassay plates containing Ery^5 and grown at 39°C for 16 hours. The individual colonies were then patched onto LB plates each containing different antibiotics (Tet^{10}, Lm^{25}, Ery^5) and grown overnight at 30°C. The colonies that grew only on LB Lm^{25} and LB Ery^5 but not LB Tet^{10} contained Tn917 insertions. These colonies were grown on CYS plates and examined microscopically for their ability to sporulate.

Transduction

Preparation of phage lysate. The donor strains, sporulation mutants HD1Mit9::Tn917 #1, 2, and 3, were grown on an LB plate containing Ery^5 at 30°C overnight (16-18 hours). Two to three colonies were used to inoculate 10 ml LB containing 0.4% glycerol. The culture was incubated at 30°C and 300 rpm to an OD_{600} of 1.6. To infect the cells with the phage CP-51ts45, two different amounts of the phage lysate, 1.2×10^6 and 1.8×10^6 plaque forming units (PFU), were added to 2×10^7 cells in 3 ml PA soft agar which was previously equilibrated to 50°C. The mixtures were then poured onto PA plates. The plates were incubated at 30°C for over 16 hours. The top agar, which contained hundreds of plaques in a lawn of cells, was collected in 6 ml of PA medium. The phage lysate was kept at 16°C for 3-4 hours, centrifuged (4,000 x g, 5 minutes, 16°C), and the supernatant was filter-sterilized using a

0.45 μ filter (VWR Scientific). The phage lysate was stored at 16°C for long term storage.

To determine the titer of the phage lysate, dilutions of the lysate were made in sterile 1% peptone and mixed with 2 x 10^7 HD1Mit9 cells. The phage/cells mixture was then mixed with 3 ml PA soft agar and poured onto PA plates. The HD1Mit9 cells were prepared using the same protocol described for preparation of the donor strain. The plates were incubated at 30°C for 16 hours and plaques were counted.

Generalized transduction. The recipient strain (HD1Mit9) was grown in 10 ml LB at 30°C for 16-18 hours. Two hundred milliliters of LB were inoculated with 1.5 ml of the overnight culture and grown at 30°C and 300 rpm to an OD_{600} of 1.3. The cells were centrifuged (7,520 x g, 4°C, 15 minutes) and resuspended in LB at a concentration of 1.3 x 10^9 colony forming units (CFU)/ml (approximately 50 fold concentrated). The transduction mixture, which contained 3x 10^8 CFU of the recipient cells and 3 x 10^8 PFU from the phage lysate, was incubated at 37°C and 250 rpm for 30 minutes. The cell/phage suspension was spread on HA Millipore membranes (Millipore Corporation, Bedford, MA), placed on LB plates containing $Ery^{0.15}$, and incubated at 37°C for 4 hours. The membranes were then transferred to LB plates containing Ery^5 and incubated at 37°C for 18-20 hours.

Cloning the Chromosomal DNA Adjacent to Transposon Insertions

The chromosomal DNA (11-14 μg) was digested with a restriction enzyme as follows. The reactions contained 30-50 units of enzyme in a 1x

reaction buffer and were incubated overnight in a 37°C water bath. The 10x enzyme buffers used were REact 2 (500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 500 mM NaCl), REact 3 (500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 1 M NaCl), and NEB 3 [500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 M NaCl]. The REact 2, REact 3, and NEB 3 buffers were used for the *XbaI*, *EcoRI*, and *BspEI* enzymes, respectively. The enzymes were inactivated by heating for 40 minutes at 70°C.

The digested DNA was ligated in a 100 µl volume using 16 units of T4 DNA Ligase and 20 µl of 5x Ligase Reaction Buffer [50 mM $MgCl_2$, 25% (wt/vol) polyethylene glycol 8000, 5 mM ATP, 5 mM DTT, 250 mM Tris-HCl pH 7.6] and incubated overnight at 16°C. The resulting ligation mixtures were used to transform 100 µl *E. coli* HB101 competent cells. The ampicillin-resistant transformed colonies were isolated after 16 hours of growth on LB agar containing Amp^{75}. The plasmid DNA was extracted from the HB101 transformants, transferred to *E. coli* DH5α or GM2163, and then analyzed by restriction endonuclease digestion. The plasmid DNA was found to be degraded and extremely unstable in the HB101 strain. To overcome this problem, the plasmids were transferred to DH5α or GM2163.

Characterization of Mutant Spores

The wild type and the mutant strains were grown in 100 ml CYS medium. The cultures were harvested 48 hours after they reached the stationary phase. The sporulated cultures were then exposed to various treatments, and serial dilutions in 0.1% peptone were plated onto LB agar and incubated overnight at 30°C. The colonies arising from the germinated spores

were counted. The resistance of the spores to heat, lysozyme, chloroform, acetone, toluene, and 1-octanol was determined as described below:

Heat. The cultures were diluted 1:10 in 0.1% sterile peptone and divided into two equal parts. One part was incubated at 55°C and the other at 65°C for 45 minutes with occasional mixing.

Lysozyme. The cultures were diluted 1:100 in 0.1% peptone containing 250 μg/ml lysozyme and incubated at 37°C for 15 minutes.

Organic solvents. The samples were treated with toluene, 1-octanol, and chloroform using the following protocol. One milliliter of the cultures was mixed with 7 ml of 0.1% peptone and 2 ml of organic solvent. The mixtures were then vortexed for 1 minute. For the acetone treatment, the cultures were diluted 1:10 in acetone and incubated at room temperature for 15 minutes.

RESULTS

To isolate a sporulation-specific gene, *B. thuringiensis* sporulation mutants were produced by transposon-mediated insertional mutagenesis. The interrupted gene was isolated, characterized, and its DNA sequence was compared to the known sporulation genes in *B. subtilis*.

The *B. thuringiensis* strain HD1Mit9 was used for transposon mutagenesis. This strain is an acrystalliferous derivative of *B. thuringiensis* subspecies *kurstaki* HD1 and contains only one 4-mDa plasmid. Most *B. thuringiensis* strains carry multiple plasmids which encode crystal proteins. Through previous studies, it was found that transposon Tn917 tends to insert into plasmids rather than the chromosome (Nicole Sinibaldi, personal communication). Using a strain that has only one plasmid increased the possibility of transposition into the *B. thuringiensis* chromosome which contains the sporulation genes.

Characterization of *B. thuringiensis* HD1Mit9

Before strain HD1Mit9 was mutagenized, its sporulation efficiency and antibiotic resistance were examined. Plasmid pLTV1, the transposon delivery vector used in this study, contained several antibiotic resistance genes which could be used to identify the colonies containing a transposon insertion. As a result, it was important to determine whether HD1Mit9 was sensitive to those antibiotics. Also, since the goal of this study was to identify a sporulation gene, HD1Mit9 was examined for its ability to sporulate. Normal

sporulation would indicate the absence of mutations in the sporulation genes.

To identify the presence of any naturally occurring antibiotic resistance, HD1Mit9 was grown on LB plates containing either Amp^{75}, Ery^{5}, Ery^{50}, Cm^{5}, Cm^{10}, or Tet^{10}. HD1Mit9 was slightly resistant to ampicillin and chloramphenicol, but sensitive to tetracycline and erythromycin. This strain sporulated well in a rich medium (CYS) after three days of incubation at 30°C.

Transformation of *B. thuringiensis* with Plasmid pLTV1

Plasmid pLTV1 (Figure 1) constructed by Camilli *et al.* (1990) was used to transform *B. thuringiensis* strain HD1Mit9. This plasmid contains transposon Tn917 which was originally isolated from *Streptococcus faecalis* (Tomich *et al.*, 1980). Tn917 has two 38 base-pair inverted repeats, represented by dark bars in Figure 1, and generates a five base-pair duplication on insertion. It encodes its transposase and confers resistance to the antibiotics erythromycin and lincomycin (Shaw and Clewell, 1985). A unique characteristic of this transposon is that transposition can be induced by a subinhibitory concentration of erythromycin such as 0.15 µg/ml (Tomich *et al.*, 1980). This transposon has been modified by addition of the following foreign DNA without affecting the transposition mechanism. It contains a promoterless *E. coli lacZ* gene which can generate transcriptional *lacZ* fusions when inserted in the correct orientation into a transcriptional unit (Perkins and Youngman, 1986). Bacteria containing such insertions turn blue on X-Gal plates. As a result, *lacZ* fusions can be used as a phenotypic screen to identify gene insertions (Youngman, 1987). A chloramphenicol acetyltransferase gene

(CAT) which can be expressed in *Bacillus* strains, an ampicillin resistance gene for use as a selectable marker in *E. coli*, and an *E. coli* origin of replication (ColEI) were also inserted into the Tn917 portion of pLTV1. Furthermore, a cluster of restriction sites, which facilitate the recovery of chromosomal DNA adjacent to Tn917 insertions, were added to the transposon Tn917.

In addition to Tn917, plasmid pLTV1 contains a tetracycline resistance gene which is expressed in both *E. coli* and *B. thuringiensis* strains. It also carries the pE194Ts temperature sensitive origin of replication which inhibits the plasmid replication at temperatures above 37°C. At elevated temperatures, pLTV1 cannot replicate and the transposon portion of the plasmid is forced into the bacterial chromosome. The resulting insertion-containing colonies are resistant to erythromycin and sensitive to tetracycline.

Plasmid pLTV1 was isolated from *B. subtilis* and introduced into HD1Mit9. However, pLTV1 was not stably maintained in HD1Mit9. Due to the presence of a different methylation process in *B. subtilis*, certain regions of the DNA might become exposed to the restriction endonucleases in *B. thuringiensis*. Therefore, the plasmid DNA may have been digested and lost from the *B. thuringiensis* cells. To circumvent this problem, it was necessary to first cycle the DNA through an appropriate strain. The plasmid was methylation-modified by replicating in *E. coli* strain GM2163 which lacks both the Dam and Dcm methylates. Previous studies done by Macaluso and Mettus (1991) indicated that the DNA isolated from GM2163 was successfully used for transformation of several *B. thuringiensis* strains. However, pLTV1 isolated from *E. coli* was also unstable in HD1Mit9. Another approach was to

transform other *B. thuringiensis* strains assuming that DNA would be easily transferred from one *B. thuringiensis* strain to another. As a result, the plasmid DNA isolated from GM2163 was transformed into *B. thuringiensis* strain Cry$^-$B. Cry$^-$B was used because it contained no plasmids. Once the plasmid pLTV1 had been placed in this strain, it could be isolated without contamination from other plasmids which are present in most *B. thuringiensis* strains. The pLTV1 DNA was then isolated from Cry$^-$B and introduced successfully into strain HD1Mit9.

The presence of plasmid pLTV1 in the HD1Mit9 transformants was confirmed by restriction enzyme digestion and PCR. The results of each experiment were analyzed by agarose gel electrophoresis. The pLTV1 DNA (1 μg) was isolated from HD1Mit9 and digested with *Eco*RI under the conditions described by the manufacturer. Restriction endonuclease analysis indicated the presence of the expected DNA fragments: 4 kb, 5 kb, and 11.4 kb (Figure 2). The PCR primers, LacNHS1 and LacNHS2 (Table 1) which were specific to the *lacZ* gene on plasmid pLTV1, amplified a 1.5 kb fragment along with a few other faint bands (Figure 3). Due to the nonspecific binding of the primers to the bacterial DNA, different size fragments were also amplified in the PCR reaction. The 1 kb DNA ladder (Gibco BRL) was run on the agarose gels as a nucleic acid molecular size standard in the enzyme digestion and the PCR experiments. HD1Mit9 and pLTV1 DNA were used as controls in the PCR experiment.

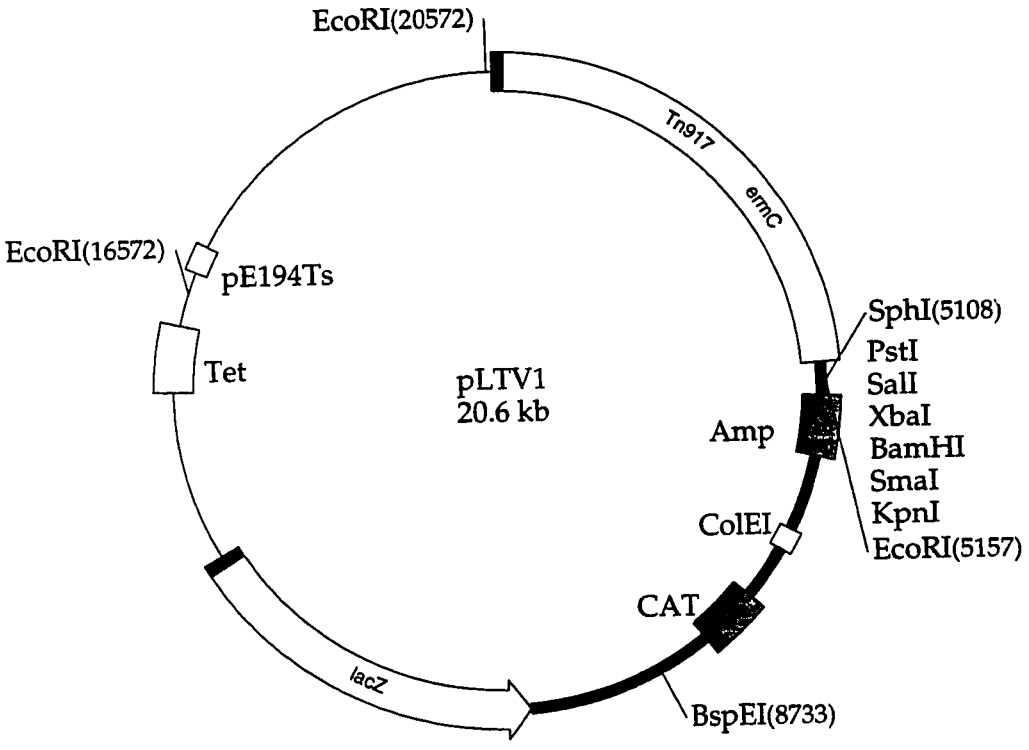
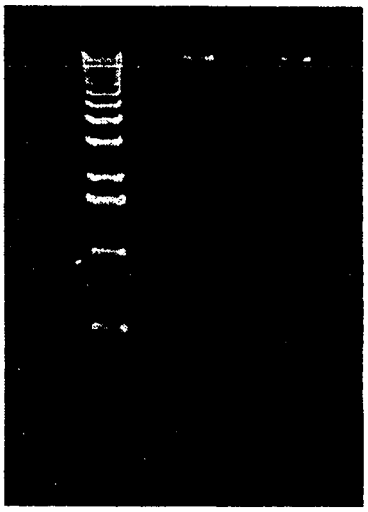


Figure 1. Map of plasmid pLTV1. The two dark bars represent the 38 base-pair inverted repeats of Tn917.

lane 1 2 3



expected bands

11.4 kb

5 kb

4 kb

lane 1 1 kb ladder

2 HD1Mit9/pLTV1

3 Cry$^-$B/pLTV1

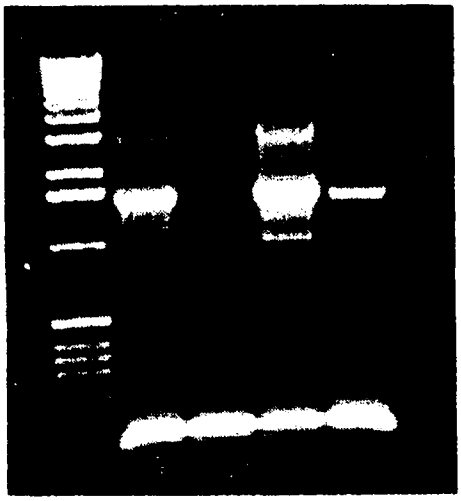
Figure 2. Agarose gel electrophoresis of plasmid pLTV1 digested with *Eco*RI. The plasmid DNA was isolated from HD1Mit9 and Cry$^-$B transformants. The 1 kb ladder contains 23 fragments at the following sizes (kb):

12.2	7.13	2.04	0.39	0.15
11.2	6.11	1.64	0.34	0.13
10.2	5.09	1.02	0.29	0.08
9.16	4.07	0.52	0.22	
8.14	3.05	0.51	0.20	

Table 1. Oligonucleotide primers used for the detection of pLTV1 in *B. thuringiensis*

Primer	Sequence (5'-3')
LacNHS1	GGCTTTCGCTACCTGGAGAGACGCGCCCGC
LacNHS2	CCAGACCAACTGGTAATGGTAGCGACCGGC

lane 1 2 3 4 5



expected band

1.5 kb

lane 1 1 kb ladder

2 pLTV1 plasmid

3 HD1Mit9

4 HD1Mit9/pLTV1

5 Cry$^-$B/pLTV1

Figure 3. Agarose gel analysis of PCR performed to confirm the presence of pLTV1 in *B. thuringiensis* HD1Mit9 and Cry$^-$B.

Stability of pLTV1 in HD1Mit9

Previous studies in *B. subtilis* indicated that pLTV1 stops replicating at very high temperatures such as 48°C (Sandman *et al.*, 1987). However, the maximum temperature used for growing *B. thuringiensis* is approximately 41°C. As a result, it was important to identify the lowest temperature at which the plasmid failed to replicate in *B. thuringiensis*. By preventing the replication of pLTV1, colonies with chromosomal insertions can be obtained.

The HD1Mit9/pLTV1 strain was grown at four different temperatures (30°C, 37°C, 40°C, 42°C) and plated onto LB $Ery^{0.05}$. Single colonies were then patched onto selective media (LB agar containing Ery or Tet). Because the erythromycin and tetracycline resistance genes on pLTV1 were lost at the nonpermissive temperatures, the colonies containing pLTV1 grew in the presence of Ery and Tet, and those that lost the DNA grew only on LB plates. The percentage of cells which retained pLTV1 was determined by dividing the number of colonies which grew on LB Ery or LB Tet by the number of colonies on LB plates (Table 2).

At temperatures above 37°C, the plasmid was extremely unstable and readily lost from the cells. Based on these results, 39°C was chosen for use in the transposition procedure because *B. thuringiensis* grew well at this temperature. The recommended temperature for growth of *B. thuringiensis* is 30°C.

Table 2. The percentage of HD1Mit9 cells containing plasmid pLTV1 at various temperatures

Temperature	% of cells containing pLTV1
30°C	96 %
37°C	10%
40°C	3%
41°C	0%

Isolation and Characterization of HD1Mit9 Sporulation Mutants

To obtain mutations in *B. thuringiensis* sporulation genes, a large population of chromosomal Tn917 insertions was prepared. During transposition, the transposon inserts in the *B. thuringiensis* genome (Figure 4). If the insertion occurs in the middle of a gene, the gene product will probably not be functional. To prepare a library of insertions, the HD1Mit9/pLTV1 strain was grown at a low temperature (30°C) in the presence of an inducing concentration of erythromycin ($Ery^{0.15}$). The culture was then diluted into a large volume of medium containing Ery^{1} and Lm^{25} and grown at a higher temperature (39°C). As indicated previously, the pE194Ts temperature-sensitive origin of replication located on plasmid pLTV1 inhibits plasmid replication at high temperatures. After growing the cells at 39°C, the majority of the surviving cells had a transposon insertion.

In the process of preparing the library, the culture was diluted several times into fresh media. This step could cause the enrichment of particular insertions in the population, making the distribution of insertions less random. In every dilution step, a small number of cells containing the transposon could be transferred and allowed to replicate. As a result, the final culture could contain a large population of cells with insertions in the same gene. To circumvent this problem, ten separate libraries were prepared to ensure that each contained different insertions.

Over 1×10^{4} colonies were screened for transposon insertions by plating onto LB plates each containing different antibiotics (Tet^{10}, Lm^{25}, Ery^{5}). Sixty-three colonies containing a chromosomal insertion were obtained (0.6%), and the average frequency of transposition was 6×10^{-3}. The

transposition frequency was calculated by dividing the number of tetracycline-sensitive colonies by the number of colonies that grew on LB Ery. The insert-containing colonies were examined microscopically for the asporogenic phenotype. The colonies were grown on CYS plates for 5-7 days and observed under the microscope for the presence or absence of spores. Only three colonies (5% of the insertion mutants) were sporulation-defective.

The asporogenic mutants were grown in minimal media to identify auxotrophs and citric acid cycle-defective colonies. If the mutants were auxotrophs or TCA cycle mutants, their sporulation-defective phenotype might be due to a metabolism problem or to the lack of energy needed for completion of the sporulation cycle. However, the three sporulation mutants (HD1Mit9::Tn917 isolates #1, 2, and 3) grew well on glucose and lactate minimal media indicating that they were not auxotrophs or defective in citric acid cycle enzymes.

To detect the expression of *lacZ* on the inserted Tn917, the mutants were grown on LB plates containing X-Gal. The *lacZ* gene codes for the enzyme ß-galactosidase which breaks the ß-galactoside, X-Gal, into its component sugars. Bacteria expressing the *lacZ* gene form blue colonies in the presence of the X-Gal substrate (Sambrook *et al.*, 1989). In this experiment, a few spots of blue color were seen in the colonies of HD1Mit9::Tn917 isolates #2 and #3. However, HD1Mit9::Tn917 #1 remained white even after four days of incubation, indicating there was no expression of the *lacZ* gene. One possible explanation for the white color phenotype is that Tn917 was inserted in such a way that the *lacZ* gene was not in the same orientation as the transcription unit and therefore was not expressed.

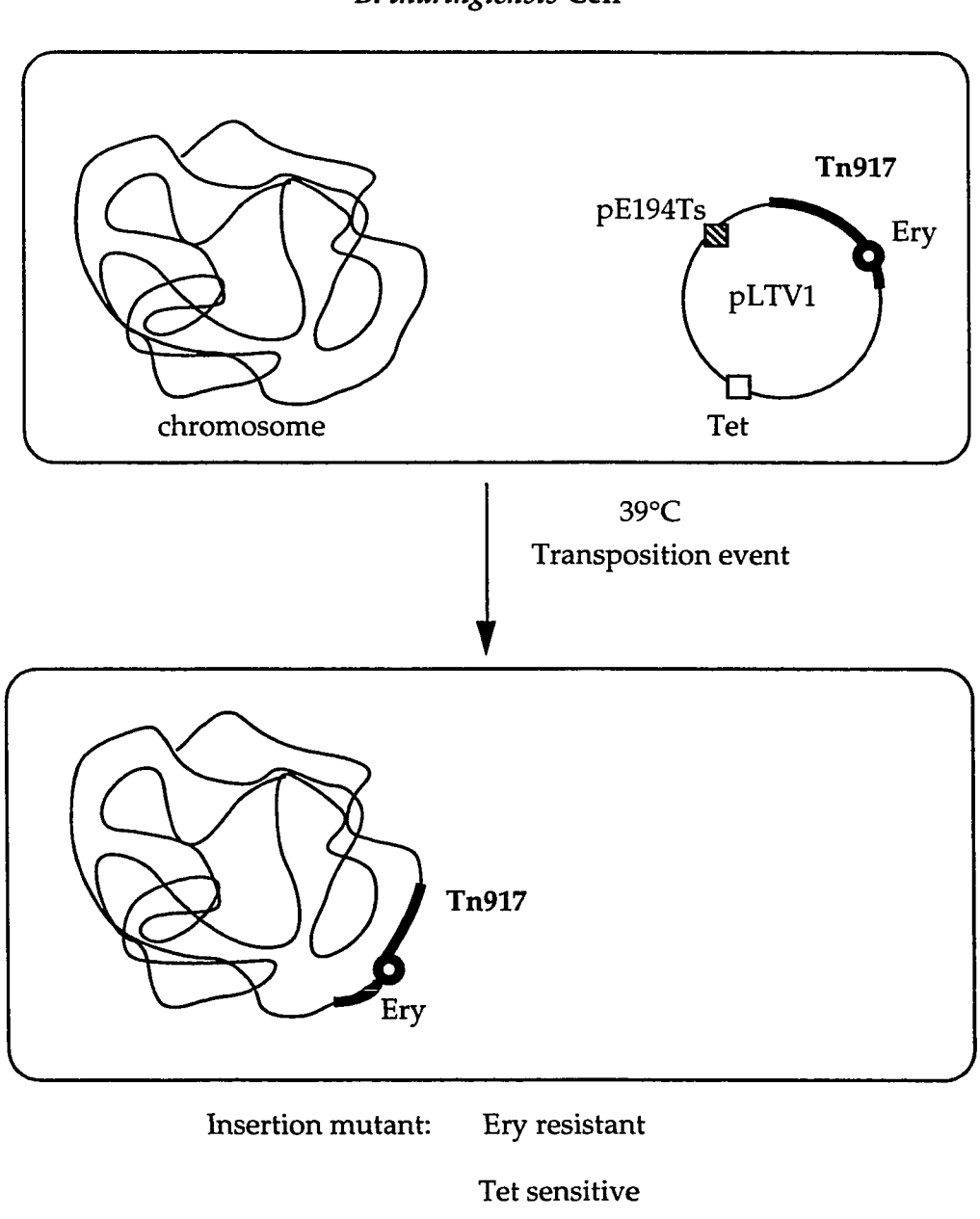


Figure 4. Transposition in *B. thuringiensis*.

Verification that Tn917 Inserted into a Sporulation Gene

Generalized transduction was used to confirm Tn917 inserted into a sporulation gene. Phage CP-51ts45 was used for the transduction experiment. This phage is a temperature-sensitive derivative of phage CP-51 which was originally isolated from soil (Thorne, 1968a). Previous studies indicated that phage CP-51 carries out generalized transduction in various *Bacillus* species such as *B. thuringiensis* (Thorne, 1978), *Bacillus cereus*, and *Bacillus anthracis* (Thorne, 1968b). The advantage of using CP-51ts45 is that the lytic and lysogenic characteristics of this bacteriophage can be controlled by altering the temperature (Curtis Thorne, personal communication). This phage undergoes a lytic cycle at 30°C and lysogeny at 37°C.

Phage lysates were prepared from the three sporulation mutants of HD1Mit9 and used to transduce the wild type HD1Mit9 strain. In this experiment, the phage was used to carry the region of the chromosome containing the Tn917 insertion into the wild type HD1Mit9 strain. Through a homologous recombination event, the DNA flanking Tn917 insertion was inserted into the homologous region of the wild type HD1Mit9 chromosome.

The erythromycin-resistant colonies obtained from transduction were grown on CYS plates for 5 days and observed microscopically for their ability to sporulate. The sporulation-defective phenotype was transferred to HD1Mit9 for only two of the mutants, HD1Mit9::Tn917 #1 and 2. This result indicated that the sporulation defect in isolates #1 and 2 was due to a Tn917 insertion in a sporulation gene. However, HD1Mit9::Tn917 #3 was a sporulation mutant possibly created by a spontaneous mutation in a sporulation gene rather than by a transposon insertion.

Isolation and DNA Sequence of the Chromosomal DNA Flanking the Tn917 Insertions

As mentioned above, unique sites in the polylinker or other regions of the transposon can be used to directly clone the DNA adjacent to transposon insertions in *E. coli* strains. *B. thuringiensis* chromosomal DNA is digested with an appropriate restriction enzyme, circularized by ligation, and introduced into *E. coli*. Plasmids obtained this way contain the gram negative origin of replication located in the transposon and the ampicillin-resistance gene which can be used as a selectable marker in *E. coli*. In addition, these plasmids carry a fragment of the *B. thuringiensis* chromosomal DNA adjacent to Tn917.

As recommended by Youngman (1987), *E. coli* HB101, which is deficient in recombination and restriction, was used for cloning. The flanking DNA adjacent to one side of the Tn917 insertions in HD1Mit9::Tn917 #1 and 2 was cloned using *Eco*RI. These DNA fragments were sequenced and compared to GenBank sequences using GCG on a VAX computer system. The sequence from HD1Mit9::Tn917 #2 showed no significant similarity to any of the sequences in the GenBank data base. However, isolate #1 showed high homology with the *B. subtilis spoVJ* gene. As a result, the chromosomal fragments on both sides of the inserted Tn917 in mutant #1 were cloned using *Bsp*EI and XbaI. The cloning strategy shown in Figure 5 was based on the method used by Youngman (1987).

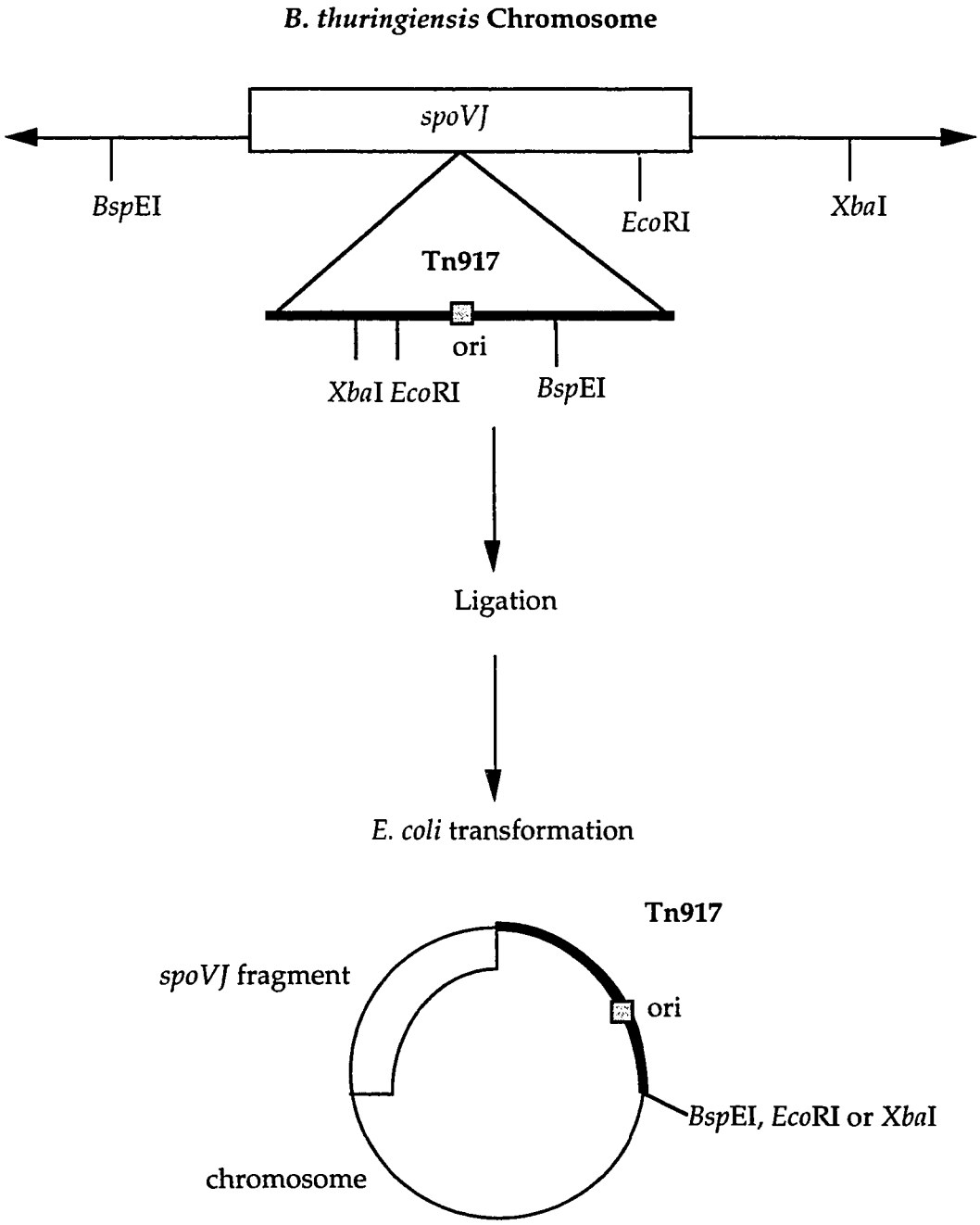


Figure 5. Cloning strategy used to isolate the HD1Mit9::Tn917 #1 sporulation gene interrupted by Tn917. The *E. coli* origin of replication (ori) is indicated by a gray square.

The plasmids obtained from *Eco*RI, *Xba*I, and *Bsp*EI digests were called pSB1211, pSB1212, and pSB1213, respectively. Plasmids pSB1211 and pSB1212 contained the *lacZ* gene and pSB1213 carried the unmodified portion of transposon Tn917 (Figures 6, 7, and 8). Plasmids pSB1211, pSB1212, and pSB1213 were digested with various enzymes (*Eco*RI, *Bgl*I, *Pvu*II, *Bsp*EI, *Hin*dIII, *Xba*I) to determine the size of the *B. thuringiensis* chromosomal fragments (Figures 9, 10 and 11). Based on these experiments, pSB1211, pSB1212, and pSB1213 contained approximately 0.47 kb, 5.66 kb, and 4.23 kb, respectively, of the chromosomal DNA flanking Tn917. These plasmids were later used for sequencing the entire cloned sporulation gene. The sequence of the NH_2-terminal coding region was obtained using plasmid pSB1213, and the COOH-terminal coding region was sequenced using plasmids pSB1211 and pSB1212.

Both strands of the cloned gene (Figures 6, 7, and 8) from HD1Mit9::Tn917 #1 were sequenced using the primers listed in Table 3. Each primer was designed based on the sequence previously obtained from the sporulation gene. The locations of the oligonucleotides are shown on the sporulation gene interrupted with transposon Tn917 in the *B. thuringiensis* chromosome in Figure 12. The sequence of the sporulation gene was compared to that of *spoVJ* in *B. subtilis*. Based on the computer analysis, the sequenced gene shared significant sequence identity with the *B. subtilis spoVJ* gene both at the DNA and protein level (Table 4). As a result, the isolated gene was believed to be the *spoVJ* gene from *B. thuringiensis*.

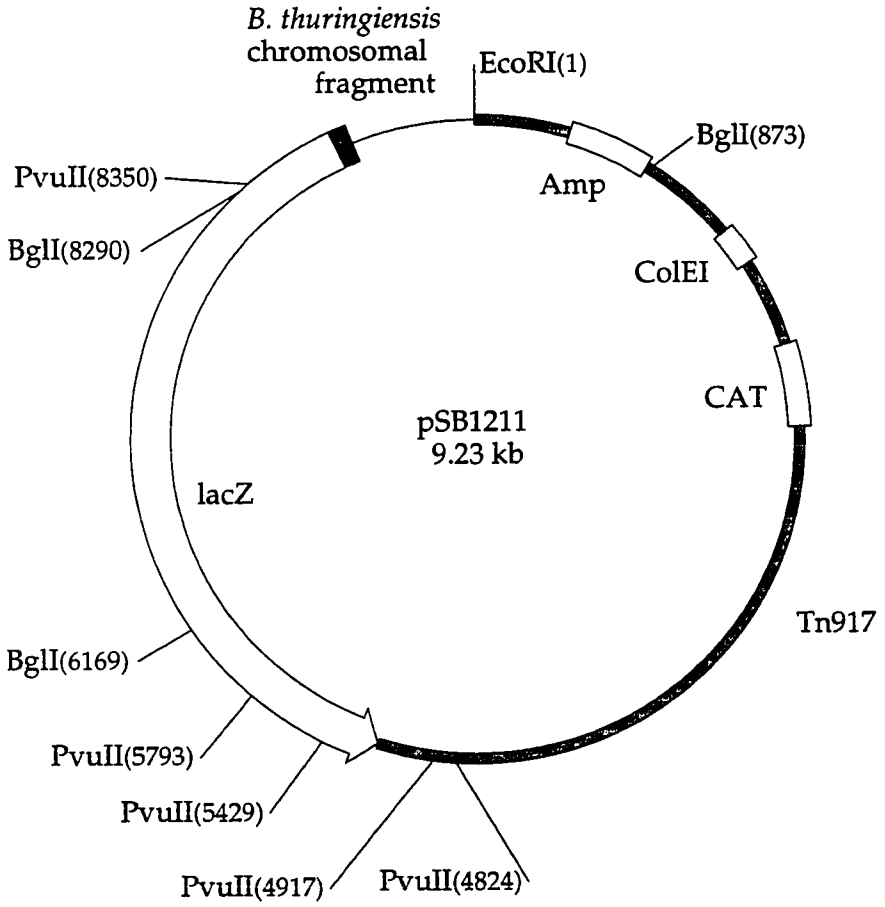


Figure 6. Map of plasmid pSB1211. The locations of the restriction sites for the enzymes used are indicated.

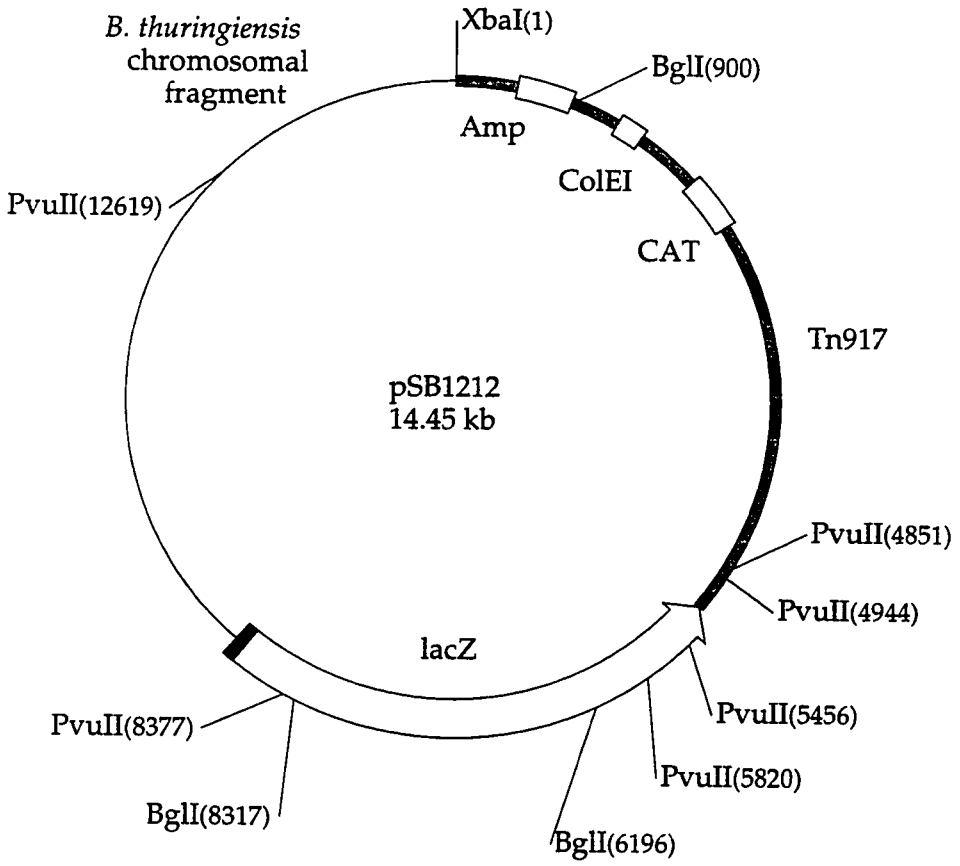


Figure 7. Map of plasmid pSB1212. The locations of the restriction sites for the enzymes used are indicated.

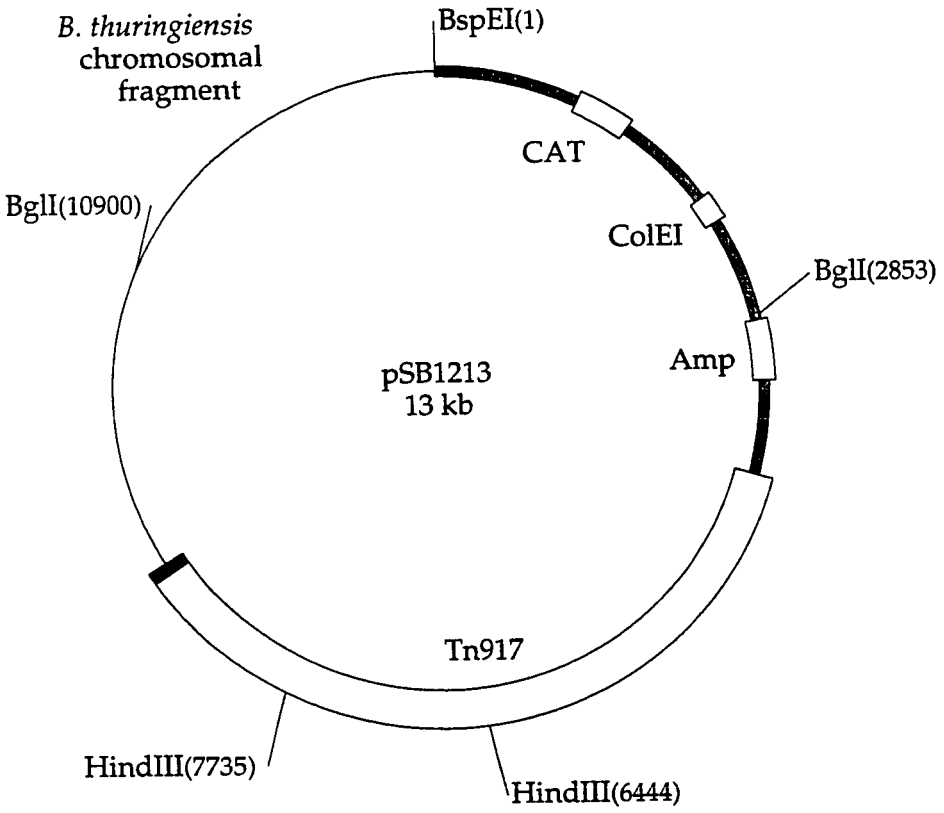
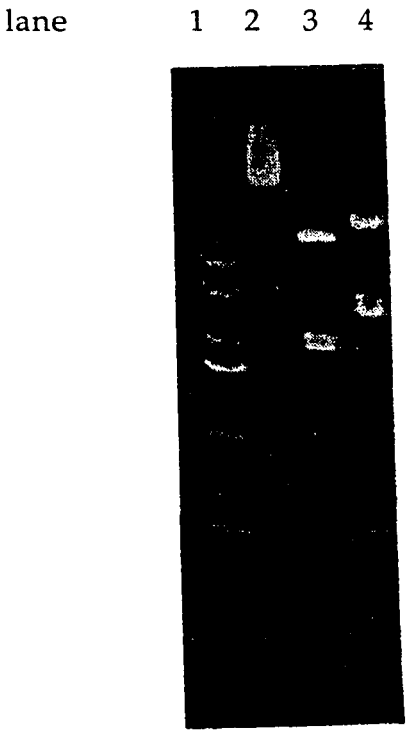


Figure 8. Map of plasmid pSB1213. The locations of the restriction sites for the enzymes used are indicated.

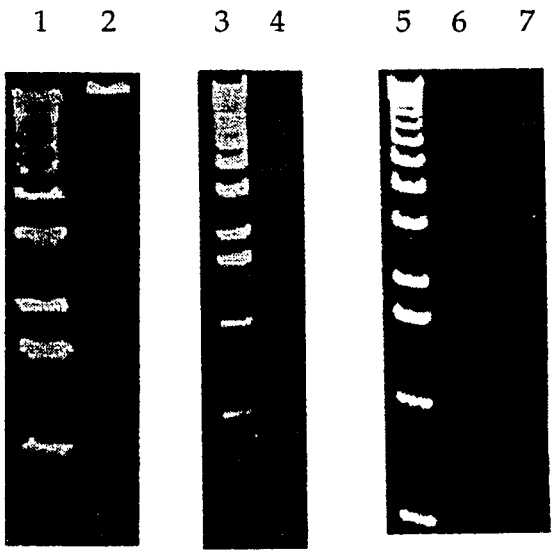


lane	1	1 kb ladder
	2	*Eco*RI
	3	*Bgl*I
	4	*Pvu*II

Figure 9. Restriction enzyme digests of plasmid pSB1211.

lane	1	*Xba*I
	2	*Bgl*I
	3	*Pvu*II
	4	1 kb ladder

Figure 10. Restriction enzyme digests of plasmid pSB1212.



lane	1	1 kb ladder
	2	*Bsp*EI
	3	1 kb ladder
	4	*Bgl*I and *Hin*dIII
	5	1 kb ladder
	6	*Bgl*I
	7	*Bgl*I and *Bsp*EI

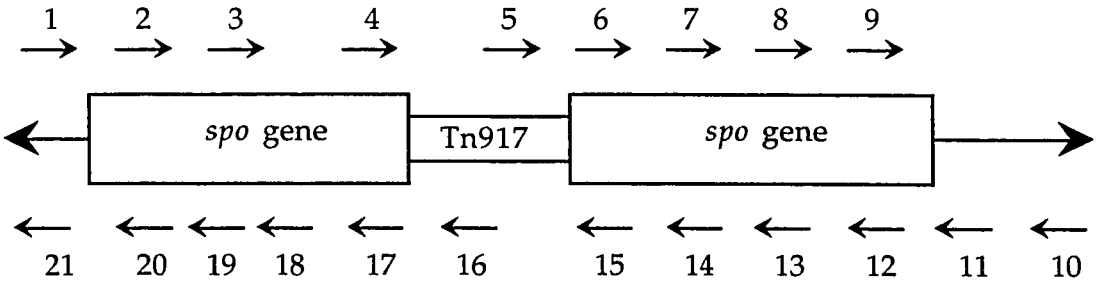
Figure 11. Restriction enzyme digests of plasmid pSB1213.

Table 3. Primers used for sequencing the *B. thuringiensis spoVJ* gene

Primer	Sequence (5'-3')
MS1	GAGAGATGTCACCGTCAAG
MS2	CCCTGTACCTGGATTCCC
MS3	GGGAATCCAGGTACAGGG
MS4	CCATCCCAACAAGCTTCCC
MS5	GGGAAGCTTGTTGGGATGG
MS6	CCTGTCCCCCTTGTAAATGC
MS7	GCATTTACAAGGGGGACAGG
MS8	CGCCGTCTACTTACAAGCAGC
MS9	GGTGGTGGGACTATGGAG
MS10	CTCCATAGTCCCACCACC
MS11A	CGAGGAGGAGAGAAGGAC
MS11rev	GTCCTTCTCTCCTCCTC
MS12A	CGAAGTGTACGGTCTGG
MS12B	CACGATGCATCG
MS13A	CGAAAGAGGCTGAATGG
MS13B	GGGCGGTATGTACGG
MS13rev	CCGTACATACCGCCC
MS14	GCATCAAATCCATACTCGATATTCC
MS14revB	CGAGTATGGATTTGATGCTCG
MS16	GGACACGATCCTAATTCAGC
MS16rev	GCTGAATTAGGATCGTGTCC

A.

Sporulation gene interrupted with Tn917



B.

primer							
	1	MS16	8	MS6	15	MS3
	2	MS14	9	MS9	16	MS8
	3	MS13rev	10	MS12A	17	MS11A
	4	MS11rev	11	MS12B	18	MS13A
	5	MS1	12	MS10	19	MS13B
	6	MS2	13	MS7	20	MS14revB
	7	MS4	14	MS5	21	MS16rev

Figure 12. (A) Location of oligonucleotides used for sequencing the *B. thuringiensis* sporulation gene (*spo* gene); (B) list of the primers used for sequencing.

Table 4. Comparison between the *B. subtilis spoVJ* gene and the cloned sporulation gene from *B. thuringiensis* HD1Mit9::Tn917 #1

Level of comparison	% identity
protein	70%
DNA	69%

The sequence of the *B. thuringiensis spoVJ* gene and the amino acid comparison between the SpoVJ in *B. thuringiensis* and *B. subtilis* are shown in Figures 13 and 14, respectively. The putative translation start codon of the *B. thuringiensis spoVJ* gene was identified based on the sequence comparison with *B. subtilis spoVJ* (Figure 13; Foulger and Errington, 1991). The putative ribosome-binding site of *spoVJ* in *B. thuringiensis* was recognized on the basis of its homology to the 3' terminal region of *B. subtilis* 16S rRNA (Figure 13; Moran *et al.*, 1982). The free energy of base-pairing between 16S rRNA and the putative *spoVJ* ribosomal binding site (determined according to Tinoco *et al.*, 1973) was -18.2 kcal. The distance between the ribosomal binding site and the putative translation initiation codon, calculated according to Moran *et al.* (1982), was 9 bases. The normal range for the free energy of base pairing in *B. subtilis* is -14 to -23 kcal, and the normal range for spacing between the ribosomal binding site and the start codon is 7-14 bases (Moran *et al.*, 1982).

The molecular mass of the putative *spoVJ* protein product was 36.7 kDa. According to Foulger and Errington (1991), the *B. subtilis spoVJ* gene encodes a 36 kDa protein. The amino acid sequence of *B. subtilis* SpoVJ contains consensus sequences present in adenine or guanine mononucleotide binding proteins (Foulger and Errington, 1991; Moller and Amons, 1985; Walker *et al.*, 1982). These sequences were also found in the *B. thuringiensis* SpoVJ protein (Figure 13). Using GCG computer analysis, a region of GC-rich dyad symmetry was found at the end of the *B. thuringiensis spoVJ* coding region. This region is most likely the stem-loop of the transcription terminator (Figure 13).

Figure 13. Sequence of the *B. thuringiensis* HD1Mit9 *spoVJ* gene (following three pages). The nucleotides are numbered on the left side of the sequence. The predicted amino acid sequence of the SpoVJ protein is shown under the nucleotide sequence using the single-letter code. Intervals of ten nucleotides are indicated by dots. The nucleotide sequence of the putative ribosome-binding site is underlined. The predicted transcription terminator stem-loop is underlined by two inverted arrows. The amino acid residues, which may be involved in nucleotide-binding, are shown in boxes.

```
                .         .         .         .         .
  1     CAGGTGAAATGAAATCTTCGTTACGAAGTGTACGGTCTGGTTGAATAGAT

                .         .         .         .         .
 51     ATCTCCATATTTTTCAATGGATTAGGAATGTTTAGAAAATGATGCATTCT

                .         .         .         .         .
101     ATTTAGTACAATAAATACACGATGCATCGTTTTTTCTGAGTAATGTCGAT

                .         .         .         .         .
151     TCGTTTTAAATCGGAAAAGTAATCTTCGTAGTCTTTTGTACAAAGTGTAG

                .         .         .         .         .
201     CCCATATATTACTGGAAGGGAGCTTTTTGTTTTTTTCTAACCAATGTCCG

                .         .         .         .         .
251     AAGTCTTCAACGTCATAAACATAACGTTTAATAGTTGAGGGTTTTCGGCC

                .         .         .         .         .
301     TTTATTCAATAAAAAAATAGAAAAGGCTTGTATTGTATCATGGAATTCCG

                .         .         .         .         .
351     TTGTCTCCATAGTCCCACCACCTTAATTATTTCTTATATTATAGCAAACT

                .         .         .         .         .
401     TTTCTGAAAATAGGCATTTACAAGGGGGACAGGAATAATAATATTTGGTG

                .         .         .         .         .
451     AGTGGATAAAATGAGGTGATTGTATGGAACAATCGATGCGAAAGAAAAAC
                                  M   E   Q   S   M   R   K   K   N

                .         .         .         .         .
501     AACAATCAAATTAATATTGTGTTAAACCATCGAAAGAAAATTTCTTTGCC
        N   N   Q   I   N   I   V   L   N   H   R   K   K   I   S   L   P

                .         .         .         .         .
551     AGCCGCAGAAAATAAAACGGTAATTTCAAATGAAACTGCAATTAAACATG
          A   A   E   N   K   T   V   I   S   N   E   T   A   I   K   H   E

                .         .         .         .         .
601     AAATGCTGCAGAGAATTGAAGAAGAGATGGGGAAGCTTGTTGGGATGGAT
            M   L   Q   R   I   E   E   E   M   G   K   L   V   G   M   D

                .         .         .         .         .
651     GATATAAAAAAGATAATAAAAGAAATATATGCTTGGATTTATGTGAATAA
        D   I   K   K   I   I   K   E   I   Y   A   W   I   Y   V   N   K

                .         .         .         .         .
701     AAAAAGACAAGAGAAGGGATTGAAGTCAGAGAAGCAAGTACTTCATATGC
          K   R   Q   E   K   G   L   K   S   E   K   Q   V   L   H   M   L
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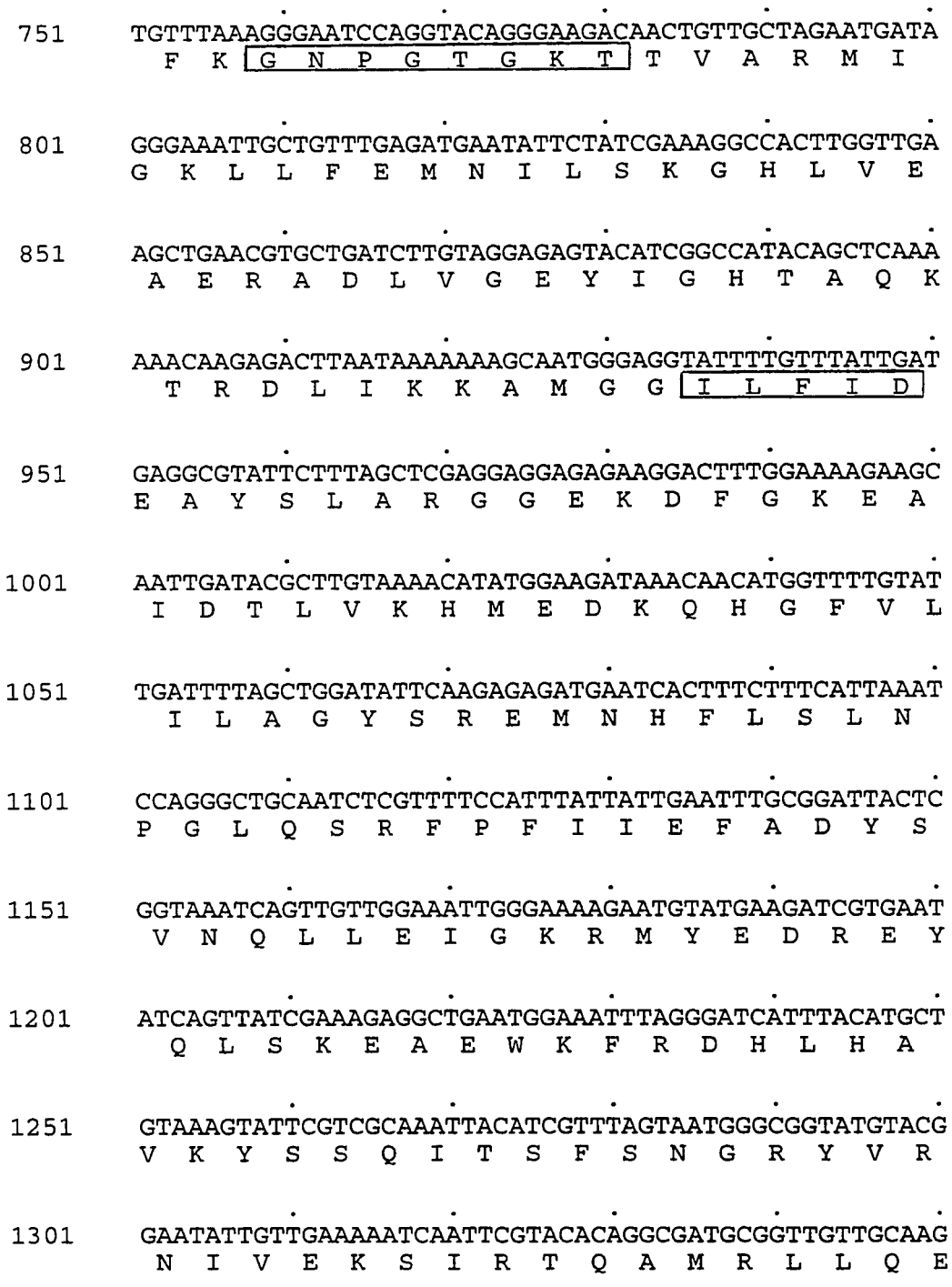
751 TGTTTAAAGGGAATCCAGGTACAGGGAAGACAACTGTTGCTAGAATGATA
F K G N P G T G K T T V A R M I
801 GGGAAATTGCTGTTTGAGATGAATATTCTATCGAAAGGCCACTTGGTTGA
G K L L F E M N I L S K G H L V E
851 AGCTGAACGTGCTGATCTTGTAGGAGAGTACATCGGCCATACAGCTCAAA
A E R A D L V G E Y I G H T A Q K
901 AAACAAGAGACTTAATAAAAAAAGCAATGGGAGGTATTTTGTTTATTGAT
T R D L I K K A M G G I L F I D
951 GAGGCGTATTCTTTAGCTCGAGGAGGAGAGAAGGACTTTGGAAAAGAAGC
E A Y S L A R G G E K D F G K E A
1001 AATTGATACGCTTGTAAAACATATGGAAGATAAACAACATGGTTTTGTAT
I D T L V K H M E D K Q H G F V L
1051 TGATTTTAGCTGGATATTCAAGAGAGATGAATCACTTTCTTTCATTAAAT
I L A G Y S R E M N H F L S L N
1101 CCAGGGCTGCAATCTCGTTTTCCATTTATTATTGAATTTGCGGATTACTC
P G L Q S R F P F I I E F A D Y S
1151 GGTAAATCAGTTGTTGGAAATTGGGAAAAGAATGTATGAAGATCGTGAAT
V N Q L L E I G K R M Y E D R E Y
1201 ATCAGTTATCGAAAGAGGCTGAATGGAAATTTAGGGATCATTTACATGCT
Q L S K E A E W K F R D H L H A
1251 GTAAAGTATTCGTCGCAAATTACATCGTTTAGTAATGGGCGGTATGTACG
V K Y S S Q I T S F S N G R Y V R
1301 GAATATTGTTGAAAAATCAATTCGTACACAGGCGATGCGGTTGTTGCAAG
N I V E K S I R T Q A M R L L Q E

```
1351    AAGATGCCTATGATAAAAATGATTTAATTGGAATATCGAGTATGGATTTG
          D  A  Y  D  K  N  D  L  I  G  I  S  S  M  D  L

1401    ATGCTCGAAGAGGAGACGCACAGTACATAAACTGTGCGTCGATTTTTGTG
        M  L  E  E  E  T  H  S  T  *

1451    TATAAGTTCGTTTACTCTTTTTTTTCTTTTTCTTGGTGTACTTCATGGAA

1501    GTGTTCCATTTTAGCGCTCTTTTCGTGTGCTGAATTAGGATCGTGTCCAA

1551    ATTGATTTACTGAGCTTTTTTGAGCTCCTTTATTAACGTGGTTTGTCATT

1601    TGTATTCACCTCACTTTAAAAATTAGTATAAACATTATATAAAGAAAAAA

1651    TCGTTAGAAAAGA
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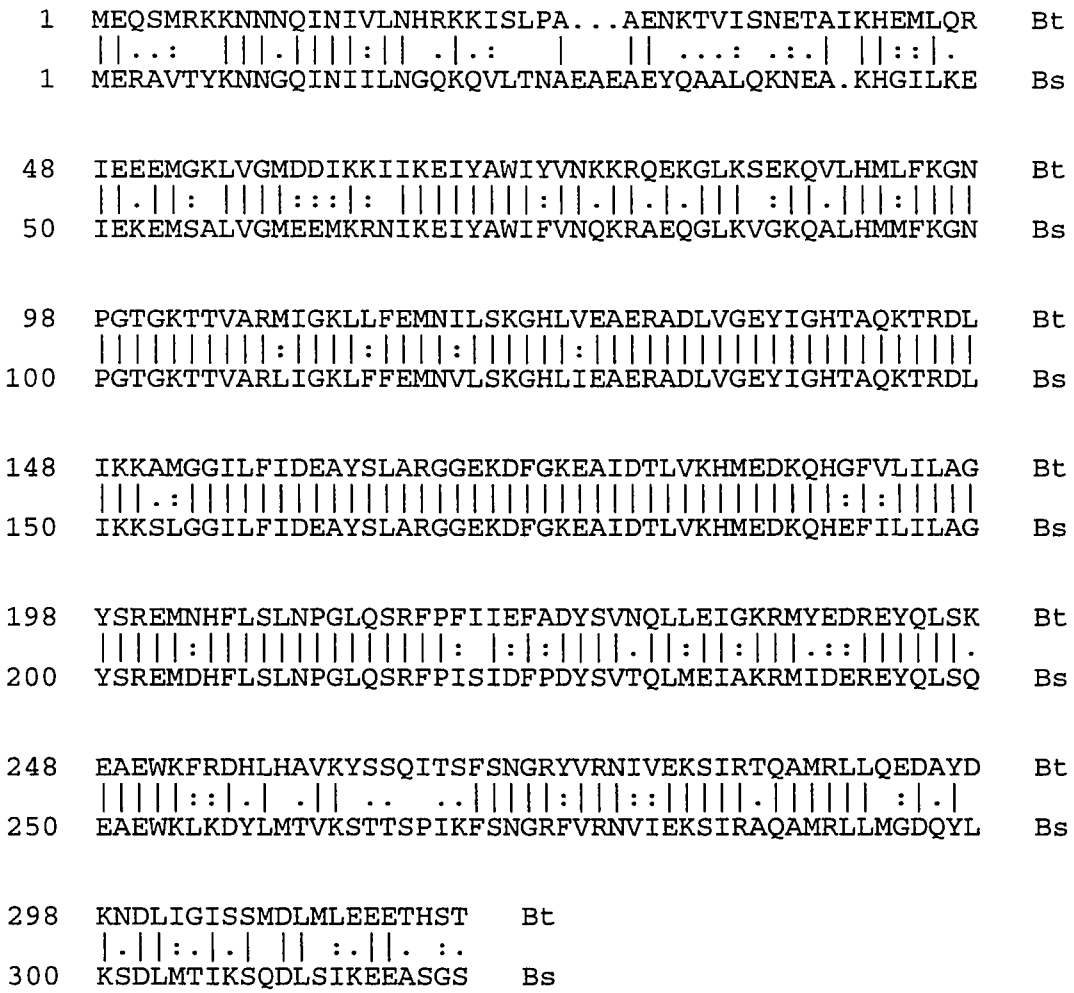


Figure 14. Amino acid comparison between the SpoVJ proteins from *B. subtilis* and *B. thuringiensis* HD1Mit9. The amino acids of *B. subtilis* (Bs) and *B. thuringiensis* (Bt) are numbered on the left side of the sequence. Identical residues are shown by a vertical line, and conserved substitutions are indicated by dots. One dot represents low similarity and two dots indicate high similarity between the two amino acids.

Growth Rate of HD1Mit9 and HD1Mit9::Tn917 #1

To determine whether the growth of HD1Mit9::Tn917 #1 was impaired as a result of the transposon insertion, the growth rate of the sporulation mutant was compared to that of wild type HD1Mit9. The *B. thuringiensis* strains were grown in CYS medium. The growth rate of the cultures was monitored by OD_{600} for 11 hours until they reached the stationary phase (Figure 15). No obvious difference was seen between the growth rate of the wild type HD1Mit9 and HD1Mit9::Tn917 #1. Both strains grew at the same rate and reached the stationary phase at approximately the same time. However, HD1Mit9::Tn917 #1 entered logarithmic growth before the wild type strain.

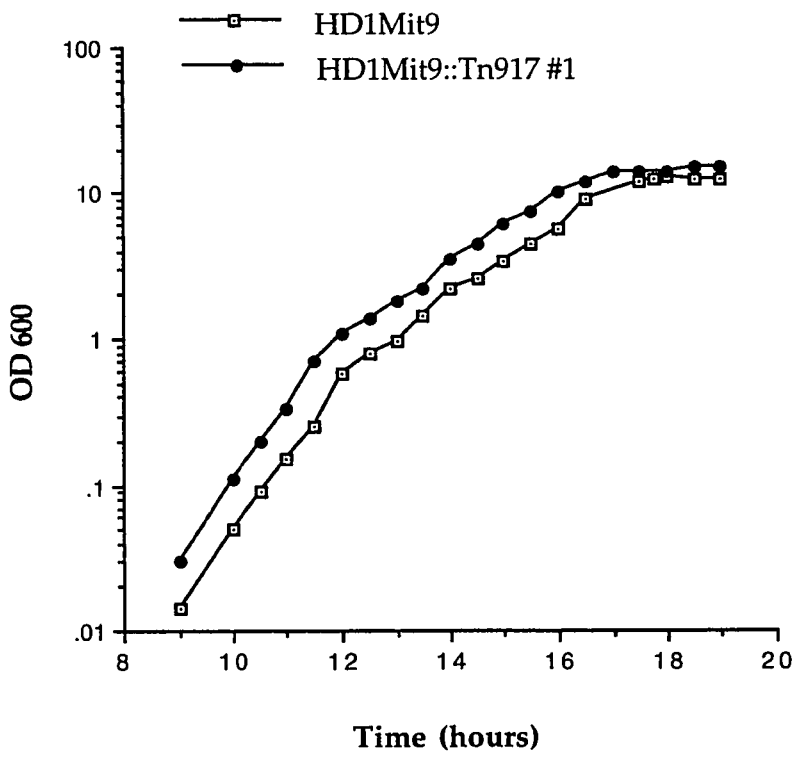
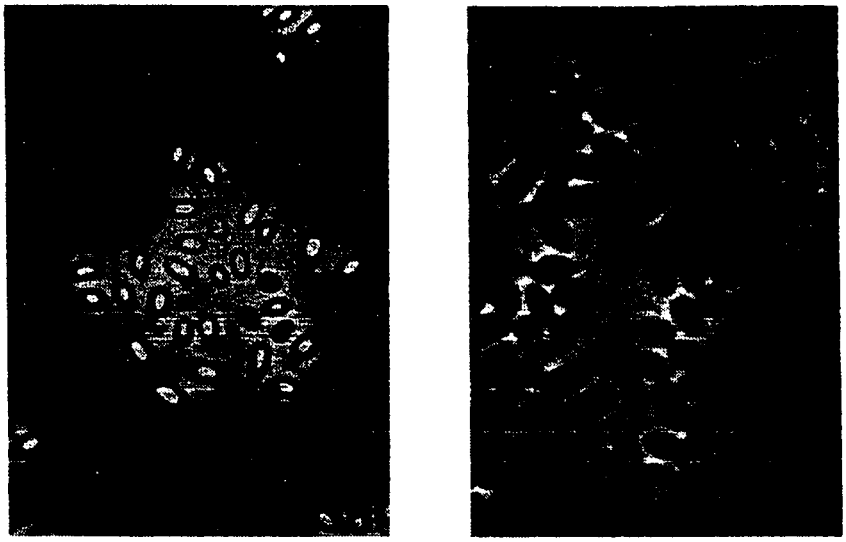


Figure 15. Growth curves of HD1Mit9 and HD1Mit9::Tn917 #1.

Characterization of the Mutant Spores

In the current study, the morphology of the spores produced by HD1Mit9::Tn917 #1 was examined using a light microscope. The mutant spores were not fully developed (immature spores) and were darker (phase gray) than the wild type spores produced by strain HD1Mit9 (Figure 16). The immature spores were also slightly oval shaped as opposed to wild type spores that were more round.

According to Hill (1983), *B. subtilis spoVJ* mutants produce immature, phase gray spores that are sensitive to heat and organic solvents and resistant to lysozyme. In these experiments, the resistance properties of the mutant and the wild type spores against heat (55°C and 65°C), lysozyme, and organic solvents (chloroform, acetone, toluene, 1-octanol) were compared (Table 5; Figure 17). The number of spores resistant to each treatment was obtained (Table 5). The percentage of resistant mutant spores was calculated by dividing the number of resistant mutant spores to the number of wild type spores (Table 5). The relative resistance of spores produced by strain HD1Mit9::Tn917 #1, from the most resistant to the least resistant, were as follows: lysozyme, heat, toluene, chloroform, acetone, and 1-octanol.



(a) (b)

Figure 16. Spores from *B. thuringiensis* HD1Mit9 (a) and HD1Mit9::Tn917 isolate #1 (b).

Table 5. Resistance properties of HD1Mit9::Tn917 #1 (mutant) and *B. thuringiensis* HD1Mit9 (wild type) spores to heat, lysozyme, and organic solvents

Treatment	Wild type spores (CFU/ml)	Mutant spores (CFU/ml)	% resistant mutant spores
55°C	3×10^8	1.6×10^3	5.3×10^{-4}
65°C	3×10^8	2.3×10^3	7.7×10^{-4}
Lysozyme	3×10^8	3.15×10^7	1.05×10
Acetone	2×10^8	1×10	5×10^{-6}
Chloroform	2×10^8	5×10	2.5×10^{-5}
1-Octanol	2.5×10^8	0	0
Toluene	2.4×10^8	1.2×10^2	5×10^{-5}

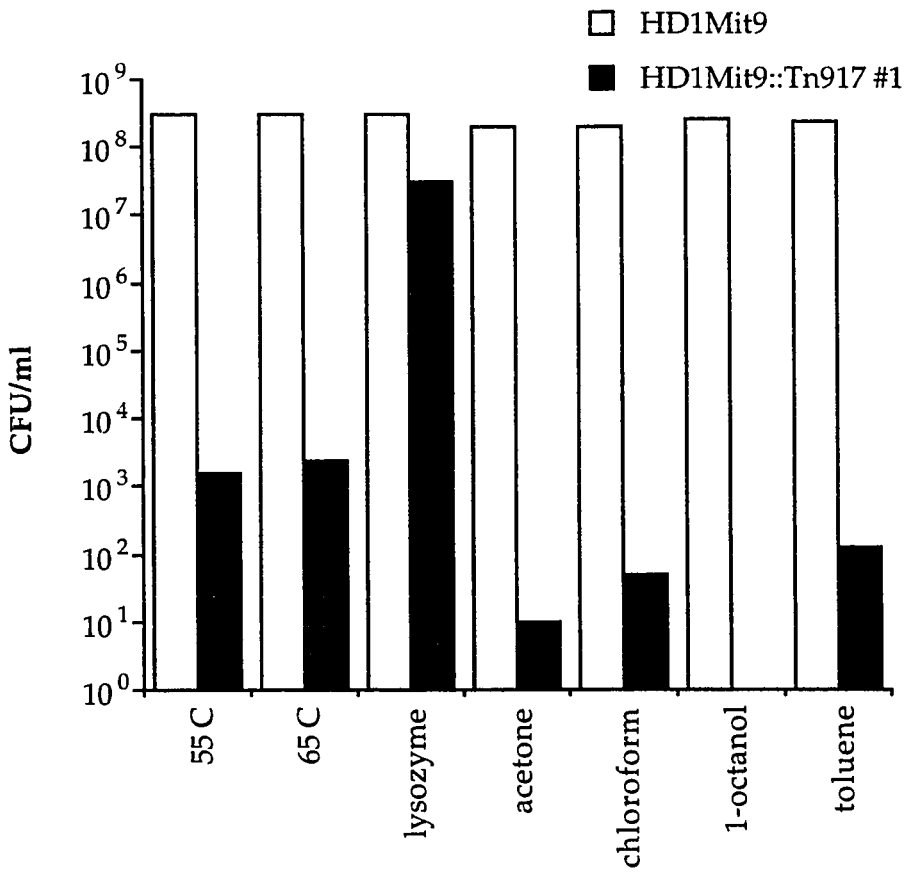


Figure 17. Resistance properties of spores from HD1Mit9 and HD1Mit9::Tn917 #1 to heat, lysozyme, and organic solvents.

DISCUSSION

Over the past 30 years, the physiological, morphological, and biochemical changes that are associated with sporulation have been studied in great detail in spore-forming bacteria such as *B. subtilis* (Errington, 1993). Some bacteria respond to poor nutritional conditions by forming a dormant cell type known as an endospore. Numerous studies have been done to identify and characterize the *B. subtilis* sporulation genes. *B. subtilis* does not contain naturally occurring plasmids, so the genes involved in sporulation are located on the chromosome. Also, it has been shown that the plasmid-cured strains of *B. thuringiensis*, including HD1Mit9 which was used in this study, undergo normal sporulation. Although most *B. thuringiensis* strains carry a number of plasmids, it is assumed that the sporulation genes in this bacterium are also scattered on the chromosome. One of the most useful approaches for isolating sporulation genes is transposon-mediated insertional mutagenesis (Youngman, 1987). The transposon Tn917 has been used widely for characterizing genes in *B. subtilis* (Sandman *et al.*, 1987), *B. megaterium* (Tao and Vary, 1991), and *L. monocytogenes* (Camilli *et al.*, 1990).

In the current study, a sporulation gene was identified in *B. thuringiensis* using Tn917 insertional mutagenesis. The method of mutagenesis used for *B. thuringiensis* was similar to the one used by Sandman *et al.* (1987) for *B. subtilis* with some modification. It was necessary to determine the best conditions to obtain efficient transposition in *B. thuringiensis*. Because of the inability of *B. thuringiensis* to grow at the high temperature used for *B. subtilis* transposition (48°C), the *B. thuringiensis* cells

were exposed to 39°C for a longer duration. This method proved effective for preparation of the *B. thuringiensis* insertion libraries. By increasing the number of times the culture was exposed to the high temperature, the plasmid pLTV1 became unstable and the transposition efficiency was increased. The *B. subtilis* culture was exposed to high temperatures two times as compared to three for the *B. thuringiensis* culture. When LB medium was used for transposition in *B. thuringiensis*, the number of transposed colonies obtained was extremely low (data not shown). Penassay broth was found to be more effective for the recovery of Tn917-insertions. However, the reason for its effectiveness is not understood. Penassay broth was also used successfully by Crawford and Streips (1990) for transposon mutagenesis in *B. thuringiensis*.

Using transposon mutagenesis, a *B. thuringiensis* sporulation gene was identified and cloned in an *E. coli* strain. The gene was sequenced and compared to the *B. subtilis* sporulation genes. Approximately 70% identity at both the DNA and the protein level was observed between the *B. thuringiensis* sporulation gene and the *spoVJ* gene from *B. subtilis*.

Several other *B. thuringiensis* sporulation genes have been sequenced and compared to *B. subtilis* sporulation genes. The Spo0F proteins of *B. thuringiensis* and *B. subtilis* share 76% sequence identity (Malvar and Baum, 1994). The *B. thuringiensis spo0A* gene and its predicted product show a high degree of similarity to the *B. subtilis spo0A* gene and its protein product (Lereclus *et al.*, 1995). The genes are 71% identical and the proteins 82% identical. Also, the amino acid sequences of the two RNA polymerase sigma factors, σ^{35} and σ^{28} from *B. thuringiensis*, have greater than 80% identity to

the *B. subtilis* sporulation-specific σ^E and σ^K, respectively (Adams *et al.*, 1991). Based on these results, the percent identity between the *B. thuringiensis* and *B. subtilis spoVJ* genes was within a normal range.

Hill (1983) indicated that the *B. subtilis spoVJ* mutant was sensitive to organic solvents and heat but resistant to lysozyme. In the current study, the resistance properties of the *B. thuringiensis* sporulation mutant against heat, lysozyme, and several organic solvents were determined. The results indicated that the mutant spores from *B. thuringiensis* showed resistance to the lysozyme treatment. However, they were significantly sensitive to heat and chemicals such as acetone, chloroform, octanol, and toluene. These results were comparable to those obtained by Hill (1983). The *B. thuringiensis* sporulation mutant, like the *B. subtilis spoVJ* mutant was oligosporogenous rather than asporogenous. It made approximately 10^3 heat-resistant spores per ml of sporulated culture as opposed to 10^8 spores per ml for wild type. This phenomenon has been observed in other stage V mutations (Hill, 1983).

Based on the sequence comparison, it was believed that the *B. thuringiensis* sporulation gene was *spoVJ*. The spore-resistance experiments showed that the *B. thuringiensis* mutant had the same phenotype as the *B. subtilis spoVJ* mutant. Taken together, these results show that the isolated sporulation gene was indeed *B. thuringiensis spoVJ*.

The function of *spoVJ* in *B. subtilis* is not fully understood. According to Foulger and Errington (1991), the presence of a nucleotide-binding motif in the *spoVJ* gene suggested that it required energy for its activity, or that it was regulated by a nucleotide. Also, Hill (1983) and Errington *et al.* (1988) have shown that the dipicolinic acid (DPA) produced by *B. subtilis spoVJ* mutants

was not incorporated into the prespore, indicating that the SpoVJ protein might be involved in the DPA transport into the prespore.

Most strains of *B. thuringiensis* produce crystal proteins during sporulation which can be used for control of lepidopteran insects. Malvar and Baum (1994) as well as Lereclus *et al.* (1995) have shown that the expression of a particular crystal gene, cryIIIA, was increased in *spo0F* and *spo0A* mutants of *B. thuringiensis*. Because of this result, it is important to gain understanding of the genes regulating sporulation. These genes could be used to develop *B. thuringiensis* strains with high insecticidal activity for biological control of pest insects.

All commercial *B. thuringiensis* strains form spores which are released into the surroundings in combination with the insecticidal proteins. These spores are resistant and can survive in the soil under extreme weather conditions. Since *B. thuringiensis* is an ubiquitous, naturally occurring soil bacterium, the release of spores into the environment is not considered unsafe. In recent years, molecular biologists have been able to manipulate *B. thuringiensis* strains to increase potency against pest insects. Many of these recombinant strains contain antibiotic resistance genes used as selectable markers. As a result, some people are not supportive of the release of recombinant strains into the environment. By identifying sporulation genes, the spore-formation pathway in the recombinant *B. thuringiensis* strains can be manipulated to produce sporulation mutant strains that are not persistent in the environment. Thus, in terms of public acceptability, an asporogenic recombinant *B. thuringiensis* strain may represent an extremely attractive alternative to other genetically engineered strains.

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